# COMPARATIVE EFFECTS OF PANTOTHENIC ACID DEFICIENCY AND INANITION ON RESISTANCE TO COLD STRESS IN THE RAT [1]

BENJAMIN H. ERSHOFF
*The Emory W. Thurston Laboratories, Los Angeles, California*

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Considerable data are available indicating that resistance to cold stress may be significantly impaired in the nutritionally-deficient animal. Thus an impaired resistance to cold has been demonstrated in rats deficient in pyridoxine (Ershoff, '51), riboflavin (Ershoff, '52a) and vitamin A (Grab and Lang, '44; Ershoff, '52b) and guinea pigs deficient in vitamin C (Dugal and Thérien, '47). Length of survival under conditions of low environmental temperature was also found to be significantly decreased for rats fed a thiamine-deficient diet (Ershoff, '50). In the present communication data are presented indicating that pantothenic acid deficiency and caloric restriction per se also impair the survival of the rat under conditions of low environmental temperature.

## EXPERIMENTAL

### *Experiment 1. Effects of pantothenic acid deficiency on resistance to cold stress*

The basal ration employed in the present experiment consisted of sucrose, 61%; casein,[2] 24%; salt mixture,[3] 5%;

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[2] Vitamin-Free test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Hubbel, Mendel and Wakeman salt mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.



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cottonseed oil,[4] 8%; and wheat germ oil,[5] 2%. To each kilogram of the above diet were added the following synthetic vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; nicotinic acid, 60 mg; biotin, 5 mg; 2-methyl-naphthoquinone, 5 mg; folic acid, 10 mg; ascorbic acid, 100 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin $B_{12}$, 100 μg; and choline chloride, 2 gm. To each kilogram of diet were also added 6,000 U.S.P. units of vitamin A [6] and 600 U.S.P units of vitamin D.[7] The vitamins were added in place of an equal amount of sucrose. Tests were conducted (1) with rats fed the pantothenic acid-free basal ration indicated above, and (2) with animals fed a similar diet supplemented with calcium pantothenate at a level of 60 mg per kilogram of ration.

For the present experiment 80 female rats of the Long-Evans strain were selected at 21 to 24 days of age and an average weight of 44.6 gm. Animals were divided into three groups. Group A (consisting of 40 rats) was fed ad libitum the basal pantothenate-free ration indicated above; group B (consisting of 20 rats) was fed ad libitum the complete ration (basal ration + calcium pantothenate); and group C (consisting of 20 rats) was pair-fed the complete ration in amounts equal to that ingested daily by rats on the calcium pantothenate-free diet. Animals were kept in individual metal cages with raised screen bottoms to prevent access to feces. Diets were made up weekly and stored under refrigeration when not in use. Rats were fed daily. All food not consumed 24 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet.

After 28 days of feeding the average body weight of the rats in the three groups was as follows: group A, 79.2 gm (range, 66 to 98 gm); group B, 143.7 gm (range, 123 to 167 gm); and group C, 112.4 gm (range, 103 to 119 gm). The

[4] Wesson.

[5] VioBin.

[6] Myva-Dry Powder, Distillation Products, Rochester, New York.

[7] HY-DEE Powder, Standard Brands, New York, N. Y.

average daily food consumption per rat during this period was 8.7 gm per day for animals in groups A and C and 12.4 gm per day for animals in group B. Two rats in group A had succumbed. Of the remaining 38 animals, 31 had become depleted of pantothenic acid as judged by stationary or decreasing body weight for a period of 5 days. All rats in groups B and C had survived (animals in group C, however, had reached a plateau in body weight).

At this time the 31 depleted rats in group A and all rats in groups B and C were selected for the following experiment. Nineteen rats in group A and 10 rats in group B were placed (one animal per cage) in a large walk-in refrigerator maintained at a temperature of $2 \pm 1.5$°C.; the remaining animals in each group remained at standard laboratory conditions ($23 \pm 2$°C.). No change was made in the diets fed. Feeding was continued ad libitum for an additional 28 days or until death, whichever occurred sooner. Twenty-four hours after the above rats were placed in the cold, all animals in group C were transferred to the cold room. No change was made in their diet. Half the rats were fed ad libitum; the remainder were fed 12 gm of diet daily (representing a caloric restriction of approximately 50% [8]). Feeding was continued for an additional 28 days or until death, whichever occurred sooner.

The findings indicate that rats depleted of pantothenic acid failed to survive under conditions of low environmental temperature. One hundred per cent of the rats depleted of pantothenic acid died during the first 72 hours of cold exposure (average survival time, 11.8 hours). Under room temperature conditions, however, 9 out of the 12 rats depleted of pantothenic acid were still alive after 28 days of feeding. All rats in group B survived, both under cold room and room temperature conditions. A significant difference was observed, how-

[8] During the first week of cold exposure the average ad libitum food consumption was 21.4 gm per day for rats in group A and 23.6 gm per day for rats in group C. For the 28-day period the average food consumption was 20.3 gm per day for rats in group A and 20.6 gm per day for rats in group C.

ever, in the caloric intake of the two groups; animals in the room temperature series ingested an average of 12.2 gm of food daily for the 28-day period, in contrast to an average intake of 19.7 gm per day for rats in the cold room series. Inasmuch as all but two of the pantothenic acid-depleted rats succumbed during the first 24 hours of cold exposure, pair feeding was not feasible for rats in group C under cold room conditions. These animals were accordingly divided into two groups, one of which was fed ad libitum while the other was fed at a level of 12 gm of diet per day. All rats fed ad libitum survived; 60% of those fed the restricted intake, however, succumbed, with an average survival time for the decedents of 19.1 days.

*Experiment 2. Effects of caloric restriction per se on resistance to cold stress*

One hundred and thirty-eight female rats of the Long-Evans strain which had been raised from weaning on a natural food ration[9] were selected at an average body weight of 142.7 gm (range, 121 to 159 gm) for the present experiment. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces and were fed ad libitum for three days the complete ration (basal ration + calcium pantothenate) employed in experiment 1. The average food consumption per rat during this period was 12.1 gm per day. After the three-day adjustment period on the new diet the animals were divided into three groups of 30 rats each and one group of 48 (average body weight per rat, 149.9 gm). Group A continued to receive food ad libitum (food consumption being determined daily for each rat). Groups B, C and D were fed the same diet but at levels of 8, 6 and 4 gm daily, corresponding to a caloric restriction of approximately $33\frac{1}{3}$, 50 and $66\frac{2}{3}$%, respectively. After 6 weeks of feeding, the average body weight of rats in the various groups was as follows: group A, 204.0 gm (range, 174 to 240 gm);

[9] Purina Laboratory Chow, Ralston Purina Co., St. Louis, Missouri.

group B, 149.5 gm (range, 132 to 163 gm); group C, 122.6 gm (range, 109 to 134 gm); and group D, 95.1 gm (range, 84 to 111 gm).

All of the rats survived the 6-week feeding period with the exception of one animal in group D. At this time the animals in the various groups were divided into the subgroups indicated in table 1. Subgroup 1 was continued at room temperature conditions at the same level of food intake the animals had received during the preceding 6 weeks. The remaining rats were placed (one animal per cage) in a large walk-in refrigerator maintained at a temperature of $2 \pm 1.5°C$. Feeding was continued for an additional 28 days or until death, whichever occurred sooner. The results are summarized in table 1.

The findings indicate that the length of survival under conditions of low environmental temperature is dependent both on the amount of calories consumed during the period of cold exposure and on the pre-test caloric intake. Under room temperature conditions 100% of the rats survived at all levels of caloric intake ($A_1$, $B_1$, $C_1$, $D_1$). Under cold room conditions, however, none of the rats survived which received less than 12 gm of diet daily. A direct correlation was observed between the pre-test caloric intake and the survival time of decedents under cold room conditions. Thus the average survival time of rats in groups $A_4$, $B_3$ and $D_3$ was 10 days, 7 days and less than one day, respectively, for animals in these groups, although they were all fed 8 gm of diet daily during the period of cold exposure. Similarly, the average survival time of rats in groups $A_5$ and $C_3$ was 8 days and three days, respectively, although both groups were fed 6 gm of diet daily during the period of cold exposure.

It is apparent from these findings that a prolonged period of caloric restriction prior to cold exposure significantly impaired the ability of rats to survive under conditions of cold stress on a caloric intake of less than 12 gm per day. When depleted rats were permitted to eat ad libitum, however, no

impairment in adjustment to cold (as measured by per cent survival and gain in body weight) was observed ($D_2$).

An interesting observation was the apparent adjustment to reduced caloric intake and the resulting increase in the efficiency of food utilization that occurred both under room

TABLE 1

*Effects of restricted caloric intake on survival under conditions of cold stress*

| GROUP | NUMBER OF ANIMALS | INITIAL BODY WT. | FINAL BODY WT.[1] | PER CENT SURVIVAL[1] | AVERAGE SURVIVAL TIME OF DECEDENTS |
|---|---|---|---|---|---|
| | | *gm* | *gm* | | *days* |
| Room temperature series | | | | | |
| $A_1$ (ad libitum)[2] | 8 | 204 | 222 | 100 | .. |
| $B_1$ ( 8 gm daily) | 10 | 149 | 181 | 100 | .. |
| $C_1$ ( 6 gm daily) | 10 | 121 | 144 | 100 | .. |
| $D_1$ ( 4 gm daily) | 9 | 95 | 104 | 100 | .. |
| Cold room series | | | | | |
| $A_2$ (ad libitum)[2] | 10 | 203 | 212 | 100 | .. |
| $A_3$ (12 gm daily) | 10 | 204 | 137 | 30 | 18 |
| $A_4$ ( 8 gm daily) | 10 | 206 | .. | 0 | 10 |
| $A_5$ ( 6 gm daily) | 10 | 203 | .. | 0 | 8 |
| $B_2$ (16 gm daily) | 10 | 149 | 145 | 100 | .. |
| $B_3$ ( 8 gm daily) | 10 | 151 | .. | 0 | 7 |
| $C_2$ (12 gm daily) | 10 | 124 | 105 | 40 | 17 |
| $C_3$ ( 6 gm daily) | 10 | 121 | .. | 0 | 3 |
| $D_2$ (ad libitum)[3] | 10 | 97 | 174 | 90 | $<1$ |
| $D_3$ ( 8 gm daily) | 10 | 96 | .. | 0 | $<1$ |

[1] After 28 days of feeding.

[2] The average ad libitum food consumption of the rats for the 28-day experimental period was 11.97 gm per day for the room temperature series and 19.18 gm per day for the cold room series.

[3] The rats in this series had an average food intake of 19.40 gm per day for the 28-day period.

temperature and cold room conditions. During the first 5 to 6 weeks of feeding in the pre-test period, a gradual and consistent loss in weight was observed in the rats in groups C and D, while body weight remained stationary for animals in group B. Subsequent to this period, however, although no change was made in the amount or composition of the diet

fed, a consistent gain in body weight occurred under room temperature conditions in every rat in the above three groups ($B_1$, $C_1$, $D_1$). It would appear from these findings that the efficiency of food utilization had improved following the 5- to 6-week period of caloric restriction. Similar results were obtained in the cold room series with groups $A_2$ and $D_2$. Both these groups were fed ad libitum. In spite of the fact, however, that virtually no difference existed in the food intake of the two groups (the average food intake of rats in group $A_2$ was 19.2 gm per day and of animals in group $D_2$, 19.4 gm per day during the period of cold exposure), the rats in group $A_2$ gained an average of 9 gm, while those in $D_2$ gained 77 gm during the 28-day period of cold exposure. It should be pointed out, however, that since the rats in group $D_2$ averaged only 97 gm in weight at the start of cold exposure, in contrast to an initial weight of 203 gm for the rats in group $A_2$, the smaller animals would have been expected to have gained more weight than the larger ones on a similar caloric intake.

### *Experiment 3. Effects of pituitary, thyroid and adrenocortical hormones on the survival time of pantothenic acid-deficient rats under conditions of cold stress*

It is well-established that requirements for adrenal cortical hormones are markedly increased under conditions of low environmental temperature (Selye and Schenker, '38; Kendall, '41; Tyslowitz and Astwood, '42) and that adrenalectomized or hypophysectomized rats fail to survive following prolonged exposure to cold. Adjustment to cold is also impaired by thyroidectomy (Leblond and Gross, '43) or administration of thiouracil (Ershoff, '48). Resistance to cold may be restored in adrenalectomized or hypophysectomized rats by administration of adrenal cortical hormone or hor-

mones or anterior pituitary extract, respectively, and in thyroidectomized or thiouracil-fed rats by injection of thyroxin. Since pantothenic acid-deficient rats also fail to survive prolonged exposure to cold, the possibility exists that the failure of such rats to withstand cold stress is due not to pantothenic acid deficiency per se but rather to an impaired production or secretion of adrenocortical (and possibly pituitary and thyroid) hormones. Recent data indicating that adrenocortical function is impaired in the pantothenic acid-deficient animal (Dumm et al., '49; Hurley and Morgan, '52; Winters et al., '52a, b; Cowgill et al., '52; Ershoff and Parrott, '52) support this hypothesis. The following experiment was undertaken to determine the effects of pituitary, thyroid and adrenocortical hormones on the survival time of pantothenic acid-deficient rats under conditions of cold stress.

The procedure and diet employed were similar to those described in connection with experiment 1. Female rats of the Long-Evans strain were placed at weaning on the purified pantothenate-free ration and selected for the following experiment at depletion (as judged by stationary or decreasing body weight for a period of 5 days). A minimum of 10 rats was employed in each experimental group.[10] All animals were continued on the pantothenate-free diet but received in addition injections of the following substances: group 1, 0.5 ml saline solution administered in divided doses twice daily; group 2, 25 μg thyroxin [11] administered once daily; group 3, 0.1 ml anterior pituitary extract [12] administered once daily;

[10] Several of the rats died while receiving injections and prior to the time when they were to be placed in the cold. These were replaced with other animals so that each group consisted of 10 rats at the start of cold exposure.

[11] Thyroxin crystals, E. R. Squibb & Sons, New York, N. Y. The thyroxin was dissolved in alkaline aqueous solution and the volume adjusted to contain 250 μg of thyroxin per milliliter.

[12] Water-soluble extract of anterior pituitary substance, The Armour Laboratories, Chicago, Illinois. Each milliliter of this extract contained 16 mg of water-soluble, heat-stable extract derived from 18.5 grains of fresh anterior pituitary substance.

group 4, 500 μg growth hormone [13] administered in divided doses twice daily; group 5, 2.5 units ACTH [14] administered once daily; group 6, 5 units ACTH administered once daily; group 7, 2.5 mg cortisone acetate [15] administered in divided doses twice daily; group 8, 5 mg cortisone acetate administered in divided doses twice daily; group 9, 2.5 units ACTH plus 500 μg growth hormone administered as above; group 10, 2.5 mg cortisone acetate plus 25 μg thyroxin administered as above; and group 11, 2.5 mg cortisone acetate plus 500 μg growth hormone. All injections were administered intraperitoneally with the exception of ACTH, which was administered subcutaneously. Rats received injections of the above substances daily for three days. On the morning of the 4th day all animals were placed (one rat per cage) in a walk-in refrigerator maintained at a temperature of $2 \pm 1.5$°C. Animals continued to receive the basal pantothenate-free ration. Injections were continued until death.

No significant difference in length of survival under cold room conditions was observed between pantothenic acid-depleted rats receiving saline solution and those administered the various hormone preparations. The average survival time in each group was less than 48 hours, with the majority of animals in each group succumbing within the first 24 hours of cold exposure. It is apparent that under the conditions of the present experiment neither thyroxin, anterior pituitary extract, growth hormone, ACTH or cortisone acetate alone, nor combinations of thyroxin and cortisone acetate, growth

[13] Growth hormone, lyophilized (lot R377237). This material was kindly provided by Dr. Irby Bunding, of the Armour Laboratories. The growth hormone preparation was dissolved in distilled water and the volume adjusted to a concentration equivalent to 1 mg of the Armour Standard 22KR2 per milliliter. The growth hormone preparation was contaminated to the extent of approximately 5 mg of TSH in terms of the new U.S.P. standard per each 50 mg of growth hormone activity.

[14] H. P. Acthar Gel (purified corticotrophin in gelatin), containing 20 Armour units per milliliter. The Armour Laboratories, Chicago, Illinois.

[15] Saline solution of Cortone Acetate, Merck and Co., Inc., Rahway, New Jersey, containing 25 mg cortisone acetate per milliliter. This material was kindly provided by Dr. R. A. Peterman of Merck and Co.

hormone and ACTH or growth hormone and cortisone acetate, were effective in prolonging the length of survival of pantothenic acid-depleted rats under cold room conditions. These findings suggest that a deficiency of one or more of the hormones indicated above was not responsible for the impaired resistance of pantothenic acid-deficient rats to cold stress. A negative result under the conditions of the present experiment, however, does not necessarily rule out the possibility that a hormonal deficiency may have contributed to the impaired resistance of pantothenic acid-depleted rats to cold stress. The injection of a hormone once or twice daily does not duplicate normal physiological conditions during which a particular hormone might be secreted more or less continuously throughout a 24-hour period. Furthermore, the possibility exists that various hormones must be present in certain ratios for optimum physiological effects, and that the hormones employed in the present experiment were inadequate in either amount or kind, or both.

## DISCUSSION

Available data indicate that rats deficient in pyridoxine, riboflavin, pantothenic acid and vitamin A have an impaired ability to survive under conditions of low environmental temperature. Present findings indicate that caloric restriction per se of a qualitatively adequate ration also impairs survival under conditions of cold stress, particularly in animals whose caloric intake was restricted prior to cold exposure. These findings suggest that the impaired survival of vitamin-deficient rats under conditions of cold stress may be due, at least in part, not to vitamin malnutrition per se but to the attendant reduction in caloric intake. In the present experiment rats depleted of pantothenic acid failed to survive under conditions of low environmental temperature (the average survival time of such animals was less than 24 hours). In contrast, 100% of the pair-fed controls on a complete ration, which were placed in the cold and permitted to eat ad libitum, survived. Even under conditions of restricted

caloric intake (approximately 50% of the ad libitum consumption), pair-fed controls survived for a prolonged period of time when exposed to a low environmental temperature (the average survival time of the decedents exceeded 19 days, with 40% of the animals still alive after 28 days of cold exposure). It would appear from these findings that restricted caloric intake was not the prime cause for the failure of pantothenic acid-deficient rats to survive under conditions of cold stress, although marked caloric restriction per se, particularly when present for a prolonged period preceding cold exposure, significantly reduced the survival time of rats under cold room conditions. It would be of interest to determine whether force-feeding a high caloric diet would prolong the survival time of pantothenic acid-deficient rats under conditions of cold stress.

Present findings indicate that pantothenic acid-deficient rats readily succumb following cold exposure (with an average survival time of less than 24 hours) but survive for a prolonged period under room temperature conditions (9 out of 12 rats depleted of pantothenic acid were still alive after 28 days of feeding). Unpublished findings from this laboratory indicate that when immature rats were placed in a cold room prior to the time they were pantothenic-acid deficient, and became depleted of pantothenic acid (as judged by stationary or decreasing body weight for a period of 5 days) after they had adjusted to the cold, they survived for an average of 21 days (range, 9 to 51 days) after depletion. It would appear, therefore, that pantothenic acid is essential for adjustment to cold, but that once such adjustment has taken place rats deficient in this factor might survive for a prolonged period under cold room conditions in the absence of dietary sources of this vitamin.

## SUMMARY

Studies were conducted on the effects of pantothenic acid deficiency on the survival time of rats under cold room (2°C.) and room temperature (23°C.) conditions. Of 19 pantothenic acid-depleted rats exposed to a low environmental tempera-

ture, 100% died within the first 72 hours of cold exposure (average survival time, 11.8 hours). Under room temperature conditions, 9 out of 12 rats depleted of pantothenic acid were still alive after 28 days of feeding. No deaths occurred after 28 days of cold exposure among rats fed a diet similar to the above but supplemented with calcium pantothenate. Although caloric restriction per se of a qualitatively adequate diet (particularly for a prolonged period preceding cold exposure) markedly reduced the survival time of rats under conditions of low environmental temperature, findings indicate that restricted caloric intake was not the prime cause of the failure of pantothenic acid-deficient rats to survive under cold room conditions.

Studies were conducted of the effects of pituitary, thyroid and adrenocortical hormones on the resistance of pantothenic acid-depleted rats to cold stress. Neither anterior pituitary extract, growth hormone, ACTH, thyroxin or cortisone acetate alone, nor combinations of thyroxin and cortisone acetate, growth hormone and ACTH or growth hormone and cortisone acetate, were effective in prolonging the length of survival of pantothenic acid-depleted rats under cold room conditions.

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# EFFECT OF VITAMINS AND NATURAL PRODUCTS ON LIVER ASCORBIC ACID OF SULFASUXIDINE-FED RATS[1]

MORTON A. SCHWARTZ AND J. N. WILLIAMS, JR
*Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*

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Recently, the authors (Schwartz and Williams, '52) have reported that a decrease in liver ascorbic acid was produced in rats fed a purified ration containing succinylsulfathiazole (sulfasuxidine). It was also found that a natural products stock ration with added sulfasuxidine was capable of reversing this effect; in fact, the liver ascorbic acid levels rose to the high levels found upon feeding the stock ration alone.

These findings have initiated the present studies, which are presented in two sections. The aim of the first section is to determine the effect of graded levels of the known vitamins, in the presence of sulfasuxidine, on liver ascorbic acid concentrations. This work is necessary to determine whether the high ascorbic acid content of the liver induced by the stock ration (Schwartz and Williams, '52) is due to the possibly higher content of the known vitamins in the stock ration or to the presence of other substances. The purpose of the second section of this work is to discover which components of the stock ration reverse to the greatest extent the depressant effect of sulfasuxidine on liver ascorbic acid.

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## EXPERIMENTAL AND RESULTS

### *The effect of graded vitamin levels plus sulfasuxidine on liver ascorbic acid*

Albino male rats[2] weighing 45 to 55 gm were employed in all of the experiments reported in this paper. The rats used in the first experiment were divided into 9 groups. Group I received a ration containing 20% casein,[3] 4% salts IV (Hegsted et al., '41), 5% corn oil, 2% vitamin mix (Williams and Elvehjem, '49; plus 1 μg of vitamin $B_{12}$ per gram of mix), 2% sulfasuxidine,[4] and 67% sucrose. Groups II–V differed from group I only in the level of the vitamin mix in the ration. Group II received the above ration with the vitamin mix increased to 10%; group III received the vitamin mix at a 20% level; group IV received the vitamin mix at a 30% level; and group V received the vitamin mix at a 35% level. With each increase in the amount of vitamin mix, the sucrose content of the ration was correspondingly decreased. Two drops of a haliver oil preparation fortified with methyl naphthoquinone and α-tocopherol (Schwartz and Williams, '52; Ramasarma, Henderson and Elvehjem, '49) were administered orally to each rat in the above groups every 4 days. Groups VI and VII were given the same rations as group I except that group VI was given the fortified haliver oil every two days, and group VII received this supplement every day.

Two additional groups were included to determine the effect of sulfasuxidine on liver ascorbic acid when it is fed with a complete stock ration. Group VIII received the natural products stock ration used in this Department (Schwartz and Williams, '52) and group IX received the same ration with the addition of sulfasuxidine at a 2% level. The fortified haliver oil was given to the rats of these two groups every 4 days. Animal weights in each group were recorded

[2] Obtained from the Holtzman-Rolfsmeyer Co., Madison.

[3] Labco.

[4] The authors wish to thank the Sharp and Dohme Co., Philadelphia, Pa., for a generous supply of sulfasuxidine.

weekly, and at the termination of the 6-week experimental period liver ascorbic acid was determined by the method of Roe and Oesterling ('44), as modified by Bolomey and Kemmerer ('46) and Bolin and Book ('47) and briefly outlined by Williams ('51).

The weight and ascorbic acid data are presented in table 1. It can be observed from this table that the rats fed the 10% vitamin mix grew better than those fed the normal 2%

TABLE 1

*The effect of various vitamin levels and a stock ration on sulfasuxidine-fed rats*

| GROUP | RATIONS [1] | NO. OF ANIMALS | 6-WEEK WEIGHT GAIN | ASCORBIC ACID IN μG PER GM OF LIVER |
|---|---|---|---|---|
| | | | *gm* | *μg* |
| I | 2% vitamin mix [2] | 8 | 154 | 218 ± 11 [3] |
| II | 10% vitamin mix [2] | 8 | 178 | 266 ± 17 |
| III | 20% vitamin mix [2] | 8 | 179 | 259 ± 10 |
| IV | 30% vitamin mix [2] | 7 | 182 | 261 ± 10 |
| V | 35% vitamin mix [2] | 8 | 174 | 242 ± 13 |
| VI | 2% vitamin mix [4] | 8 | 165 | 256 ± 14 |
| VII | 2% vitamin mix [5] | 8 | 153 | 258 ± 14 |
| VIII | Stock [2] | 8 | 198 | 362 ± 5 |
| IX | Stock + sulfasuxidine [2] | 8 | 177 | 391 ± 12 |

[1] Detailed description of each ration is given in the text. Each ration except that of group VIII contained 2% sulfasuxidine.

[2] Fortified haliver oil given at the rate of two drops every 4 days.

[3] Standard error of the mean.

[4] Fortified haliver oil given at the rate of two drops every two days.

[5] Fortified haliver oil given at the rate of two drops every day.

level of vitamins. However, increasing the vitamin mix level above 10% had little influence on the weight gain. The stock ration (group VIII) induced the greatest growth, as found before (Schwartz and Williams, '52) and the addition of sulfasuxidine to the stock ration caused a decline in the weight gain.

With respect to liver ascorbic acid, this table shows that raising the level of the vitamin mix in the ration to 10% produced a considerable increase in liver ascorbic acid (compare groups I and II). This has also been found previously

(Schwartz and Williams, '52). However, a further increase in the vitamin mix content of the rations to a 20, 30 or 35% level produced no significant change in liver ascorbic acid as compared to the group receiving the 10% vitamin mix (compare groups III–V with group II). These results indicate that the maximum prevention by the water-soluble vitamins of the depressant action of sulfasuxidine on liver ascorbic acid was given by the ration containing 10% vitamin mix.

Analogous results were obtained when the frequency of administration of the fat-soluble vitamins was varied while the vitamin mix content of the rations was maintained at a constant 2% level. A significant increase in liver ascorbic acid resulted from increasing the rate of administration of the fortified haliver oil from two drops per rat every 4 days to two drops per rat every two days (compare groups I and VI). However, no further effect on liver ascorbic acid was obtained by giving the fortified haliver oil daily (compare groups VI and VII).

The ability of the stock ration to prevent the depressant action of sulfasuxidine on liver ascorbic acid is also presented in table I. It can be observed that sulfasuxidine, when fed in the stock ration, did not produce a reduction in the liver concentration of ascorbic acid. Moreover, there was a somewhat higher level of liver ascorbic acid in the group which received both the stock ration and sulfasuxidine (compare groups VIII and IX).

### *Effect of the various components of the stock ration on liver ascorbic acid*

In these experiments the various components of the stock ration employed in the preceding experiments were added to a purified ration in the same concentrations in which they were present in the stock ration. The group II ration of the previous experiment — i.e., that containing 10% vitamin mix plus 2% sulfasuxidine — was used as a base for each component-containing ration. As reported above, the vitamin

content of this ration is such that any additional amounts of the water-soluble vitamins supplied to the ration by the stock ration component would have no further effect on liver ascorbic acid. Therefore, the stimulatory action of other agents in the stock ration components on liver ascorbic acid levels could be studied. Also, to serve this purpose the fortified haliver oil was given to each rat every two days.

Since the protein content of the rations containing the stock ration components was increased to some extent by the added protein of the component, the first three groups were set up to determine the effect of various levels of dietary protein on liver ascorbic acid concentrations. Group A received the same ration as that fed group II of the preceding section; group B received a similar ration except that the casein level was increased to 25%; and group C received a similar ration containing 30% protein. Group D was fed a complete stock ration plus 2% sulfasuxidine, which is the same ration given to group IX of the preceding section. The remaining groups received the ration of group A to which the individual components of the stock ration were added. To that of group E was added 28% fine ground wheat, to that of group F, 24% fine ground yellow corn, to that of group G, 10% soybean oil meal and 10% linseed oil meal, to that of group H, 8% alfalfa meal, that of group J, 12% powdered skim milk, and to that of group K, 5% melted butter. It was not considered necessary to test either the salt fractions or the liver concentrate fraction [5] of the stock ration, since an adequate salt mixture has always been included in the purified rations and the effect of the liver concentrate has already been reported (Schwartz and Williams, '52).

Animal weights were recorded weekly. At the termination of the 6-week feeding period, liver ascorbic acid was determined by the modified Roe and Oesterling method. Also, a macro-Kjeldahl technique was employed for the determination of nitrogen in each of the rations.

[5] Wilson's 1: 20.

The results of the ascorbic acid analyses and the protein determinations, and the 5-week weight gains, are presented in table 2. With respect to ascorbic acid, the results indicate that an increase in the casein content of the purified rations to 30% casein or 28.3% of Kjeldahl-determined protein ($N \times 6.25$) is associated with a corresponding increase in liver ascorbic acid (compare groups A, B, and C). However, the complete stock ration (group D), although containing only 22.2% of determined protein, again produced the greatest concentrations of liver ascorbic acid. Groups E and F, which received the wheat and yellow corn components, respectively, showed the highest liver ascorbic acid values of any group receiving a component-containing ration. Therefore there appears to be no consistent relationship between high liver ascorbic acid levels and the protein content of the ration. For example, the protein content of the ration of group G was found to be 27.2% and the liver ascorbic acid value for this group was almost identical with that of group H, which received a ration containing 21.0% protein. Concentrations of liver ascorbic acid similar to those of groups G and H were also found for group J. The ascorbic acid values of these three groups, however, were much lower than the values of groups E and F. The least increase in liver ascorbic acid over the group A level was shown by group K, which received 5% melted butter component; and, as expected, the protein content of the two rations were almost identical.

From the weight data of table 2 it can be seen that an increase in the casein content of a sulfasuxidine-containing ration from 20 to 25% produced a considerable increase in weight gain, while an additional increase in casein to 30% produced no further effect. It is interesting to note that the best growth was exhibited on the purified ration plus soybean oil meal and linseed oil meal (group G), rather than by the group fed the complete stock ration (group D). The data also indicate that there is little or no consistent relationship between weight gain and liver ascorbic acid.

TABLE 2

*Effect of various protein levels and stock ration components on sulfasuxidine-fed rats*

| GROUP | RATIONS [1] | NO. OF ANIMALS | 5-WEEK WEIGHT GAIN | ASCORBIC ACID IN $\mu$G PER GM OF LIVER | PROTEIN CONTENT OF THE RATION |
|---|---|---|---|---|---|
| | | | *gm* | *$\mu$g* | % |
| A | 20% casein | 8 | 149 | 289 ± 10 [2] | 19.8 |
| B | 25% casein | 8 | 191 | 327 ± 7 | 23.8 |
| C | 30% casein | 7 | 192 | 359 ± 9 | 28.3 |
| D | Stock | 7 | 188 | 425 ± 14 | 22.2 |
| E | Purified ration + wheat [3] | 8 | 202 | 370 ± 12 | 23.6 |
| F | Purified ration + corn [3] | 7 | 197 | 388 ± 12 | 22.4 |
| G | Purified ration + soy bean oil and linseed oil meal [3] | 8 | 226 | 326 ± 10 | 27.2 |
| H | Purified ration + alfalfa [3] | 7 | 179 | 323 ± 12 | 21.0 |
| J | Purified ration + milk [3] | 8 | 187 | 327 ± 10 | 23.1 |
| K | Purified ration + butter [3] | 8 | 154 | 317 ± 10 | 19.6 |

[1] Detailed description of each ration is given in the text. Each ration contains 2% sulfasuxidine.

[2] Standard error of the mean.

[3] Each of these components was added to the 20% casein ration containing 2% sulfasuxidine (group A ration).

## DISCUSSION

From the experimental results it appears that some factor, or factors, in the stock ration other than the vitamin or protein content is responsible for the high liver ascorbic acid concentrations found in our experimental animals. However, the lack of correlation between the protein content of a ration and its elevation of liver ascorbic acid levels does not rule out the possibility that specific amino acids contribute to the increase in ascorbic acid. It is also possible that some substance similar to the various organic compounds found by Musulin et al. ('39) to be active in stimulating ascorbic acid synthesis may be the active principle in the stock ration. These authors (Longenecker et al., '39) reported finding such stimulatory activities in oats and alfalfa, but not in wheat. However, our results indicate that alfalfa is one of the least active stock ration components in inducing high ascorbic acid levels in rat liver, while wheat is one of the most active components.

Sulfasuxidine is known to have bacteriostatic properties (Welch and Wright, '43; Miller, '45; Gant et al., '43; Poth et al., '42; White, '42). This suggests the possible involvement of intestinal microorganisms in the maintenance of liver ascorbic acid. Sulfasuxidine and the stock ration may produce their respective depressed and elevated liver ascorbic acid levels by affecting the bacterial synthesis of some factor necessary in the synthesis of ascorbic acid by the rat. It is also possible that more than one agent may be involved in the stock ration effect on liver ascorbic acid: one factor which prevents the depressant action of sulfasuxidine and another which induces high levels of liver ascorbic acid.

At the present time investigations are proceeding on the nature of the factor, or factors, in the stock ration which is able to reverse the depressant action of sulfasuxidine and to elevate ascorbic acid concentrations in rat liver.

SUMMARY

1. A complete stock ration composed of natural products has been found to reverse the depressant action of sulfasuxidine on the maintenance of liver ascorbic acid in the rat.

2. The nature of the effect of various amounts of the known vitamins on liver ascorbic acid has been determined.

3. The possibility that the effect of the stock ration is due to increased quantities of the known vitamins has been ruled out since the natural products ration still produces considerably higher levels of ascorbic acid than a purified ration containing excess amounts of the known vitamins.

4. The various components of the stock ration have been tested for their effect on liver ascorbic acid maintenance. The components giving the greatest effect were wheat and yellow corn, although certain other components of the natural ration stimulated ascorbic acid levels significantly.

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# EFFECTS OF A FAT-FREE DIET ON GROWING FEMALE RATS, WITH SPECIAL REFERENCE TO THE ENDOCRINE SYSTEM

THEODORE C. PANOS AND JOHN C. FINERTY

*Departments of Pediatrics and Anatomy, University of Texas Medical Branch, Galveston*

ELEVEN FIGURES

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The earliest experimental evidence that dietary fat has specific biologic importance was included in preliminary reports by Evans and Burr ('27a, b, '28), in which attention was drawn to the retardation of growth and disturbance of ovulation in rats fed a fat-free ration. Subsequently, Burr and Burr ('29, '30) conclusively demonstrated that growing rats deprived of fat regularly develop a "deficiency syndrome" characterized by growth failure, skin and kidney changes, irregular ovulation and impaired reproduction. They further established that this syndrome is due to the absence in the diet of unsaturated fatty acids, specifically linoleic and arachidonic acids, and proposed the concept of "essential fatty acids." These studies provided the impetus for a sustained surge of research activity, but, in spite of the implications of the original work, there have been relatively few studies of the interrelationships between fat-free diets and endocrine function.

The purpose of the investigations here reported was to delineate the "spectrum" of effects produced by the prolonged feeding of a diet devoid of fat to growing rats, with particular reference to the endocrine glands. It was felt that careful scrutiny of concomitant changes was an important prerequisite for fundamental understanding of the problem. The

numerous advances in nutrition during recent years have made such an approach particularly desirable, inasmuch as the preparation of adequate purified diets has been placed on a considerably more reliable basis than was possible in early studies. In addition to the usual observations of the effects on growth, skin changes, and ovulation, an attempt was made to study the gross and microscopic characteristics of all the endocrine organs, the liver, heart and kidney. Spectrographic analyses of fatty acids of the blood, skin, liver, heart and kidneys will constitute a separate report.

## MATERIALS AND METHODS

Female rats of the Holtzman strain were obtained at weaning, the average weight at the beginning of the experiment being 54 gm. One hundred rats were placed on the fat-free diet and 16 controls were fed laboratory chow.[1] Access to urine and feces was prevented by use of false-bottom cages, each of which contained 5 animals. Distilled water was supplied for drinking. Air-conditioning was installed early in the experiment, and except for the first 4 weeks, temperature and humidity were maintained within a fairly constant range, although the latter was consistently high (about 70%).

The percentage composition of the fat-free ration [2] was as follows: Fat-free, vitamin-free casein [3] 18; sucrose 76; salts 4; and cellulose 2. Vitamins were added in the following amounts per kilogram of feed: thiamine chloride 5 mg, riboflavin 10 mg, pyridoxine 5 mg, calcium pantothenate 50 mg, nicotinic acid 25 mg, para-aminobenzoic acid 10 mg, biotin 2 mg, inositol 1,000 mg, folic acid 2 mg, $B_{12}$ 1.8 mg,[4] menadione 50 mg, crystalline vitamin A acetate 1 mg, crystalline vitamin $D_2$ [5] 20 mg, and alpha-tocopherol 100 mg. The last three vitamins were

[1] Purina.

[2] We are indebted to Dr. Wendell H. Griffith, Professor of Biochemistry and Nutrition, University of California at Los Angeles, who designed this diet for us.

[3] Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[4] Kindly supplied by Dr. Augustus Gibson, Merck and Co., Inc., Rahway, New Jersey.

[5] Drisdol®.

combined in a special "mix," which was made up in small amounts at frequent intervals: the vitamin A acetate was dissolved in petroleum ether, the alpha-tocopherol in ethyl ether and these two and the vitamin $D_2$ were added to sucrose which was then thoroughly stirred until dry. The complete ration was mixed in amounts which would last 5 to 7 days. Food containers were improvised from 8-ounce ointment jars, the caps of which had a central punched-out area, 32 mm in diameter, to allow access to food and to minimize spillage.

Food intake was not specifically measured, inasmuch as previous studies have indicated that animals on a fat-free diet consume as much food as controls, in spite of their smaller size (Burr and Burr, '30; Maeder, '37). Gross estimations of intake yielded the impression that rats on the experimental diets were voracious eaters, but no comparison with the amount taken by the control animals was attempted. Food was readily accessible, was replenished daily, and an excess of food was always present. Therefore we believe that little, if any, of the difference noted between groups can be accounted for by failure to eat as much food.

The animals were weighed weekly as the only means employed to evaluate growth. Vaginal smears were done in large groups throughout the course of the experiment. Examination for skin changes was made during the process of the weekly weighing. Criteria employed for evaluating the severity of change were as follows:

*Tail — minimum:* erythema, ridging or early scaling, or both, within one inch of tip of tail;

\+ : extension of erythema, ridging and scaling to involve more than one inch of end of tail or base of tail, or both;

\+ + : ridging, scaling and oozing along entire course of tail with occasional punctate hemorrhages;

\+ + + : severe ridging and exudation with numerous punctate and ridge hemorrhages.

*Feet — minimum:* erythema and scaling between toes;
+ : extension of erythema and scaling to involve lateral borders of feet and the ankle;
++ : scaling, oozing and occasional hemorrhagic areas over entire dorsum of foot and along ankle; occasionally edema of foot;
+++: thick crusting and oozing with hemorrhages and edema.

Changes in the pelt were recorded only as loss of hair, since no dandruff was observed.

After 24 weeks, 30 of the animals receiving the fat-free ration and 10 of the control group, receiving chow, were sacrificed. The remaining fat-deficient rats were separated by lot and placed in 4 dietary groups: fat-free diet; fat-free diet plus 20 mg methyl linoleate daily; fat-free diet plus 40 mg methyl linoleate daily; and laboratory chow [6] diet. The methyl linoleate [7] was obtained in 10-gm ampoules. A mixed tocopherol preparation [8] was added for anti-oxidant effect in the proportion of 100:1 linoleate to tocopherol. The resultant mixture was found to contain 20 to 21 mg methyl linoleate per drop, and the daily dose was administered by dropper directly into the mouth of the rat. The linoleate preparation was stored at all times in the freezing compartment of the refrigerator and was removed once daily only for the period required for administration. Sixteen days after storage of a linoleate sample in the refrigerator, the iodine number was found to be practically identical with that of the original determination. Spectral analysis of the fat extracted from the laboratory chow revealed that it contained an average of 2.0% of unsaturated fatty acid, most of which was linoleic acid. On the basis of an average consumption of 8 gm chow per

[6] See footnote 1, page 398.
[7] Purchased from Hormel Institute, Austin, Minnesota.
[8] Natopherol (R), Abbott Laboratories, North Chicago, Illinois.

day per rat, the animals maintained on chow received 135 mg linoleic acid per rat per day.

Oxygen consumption rates were measured in animals from each group a few days before sacrifice. These measurements were made primarily as an additional check on the production of fat deficiency. It has previously been demonstrated (Wesson and Burr, '31; Burr and Beber, '37; Burr, '42) that the metabolic rate is increased in fat deficiency. A round desiccator jar, 10 inches in diameter, to which was attached a spirometer, was employed. The animals were fasted 8 to 10 hours before being placed in the jar, and duplicate readings were taken only during sleep or absolute rest. Following correction to standard barometric pressure and temperature, the final results are expressed as milliliters of $O_2$ consumed/100 gm body weight/hour.

Ten weeks after separation into the dietary supplement groups (34 weeks after the start of the experiment), the rats were sacrificed. The animals were weighed and placed in an ether jar. After deep narcosis had been achieved, blood for fatty acid studies was obtained by direct heart puncture. Skin specimens from the ventral thorax and dorsal interscapular area were taken, after removal of hair by fine clippers. The following organs were weighed on a torsion balance immediately upon removal: pituitary, thyroid, thymus, adrenals, ovaries, uterus, liver, heart and kidneys; a portion of the pancreas was also taken. The pituitary was fixed in Zenker-formol solution and prepared for histological study by the technique of Briseno-Castrejon and Finerty ('49). A portion of the liver and one adrenal were fixed in 10% formalin and stained with Sudan IV. The remaining tissues were fixed in Bouin's solution for routine hematoxylin-eosin staining. Most of the liver, heart and kidneys were set aside for tissue fatty acid studies. All tissues were sectioned at 6 μ except the hypophysis, which was sectioned at 4 μ.

Differential cell analysis of the anterior pituitary glands was made by counting all the acidophiles, basophiles and total nuclei in every 10th microscopic field in a centrally located

horizontal section, under oil immersion and using 20 × oculars. Cellular percentages were calculated on counts of approximately 1,000 cells in each gland.

## RESULTS

### *Effect on growth*

Impairment of growth was apparent within two weeks and the maximum weight achieved by the fat-deficient animals was 80% of that of the controls (fig. 1). Those rats

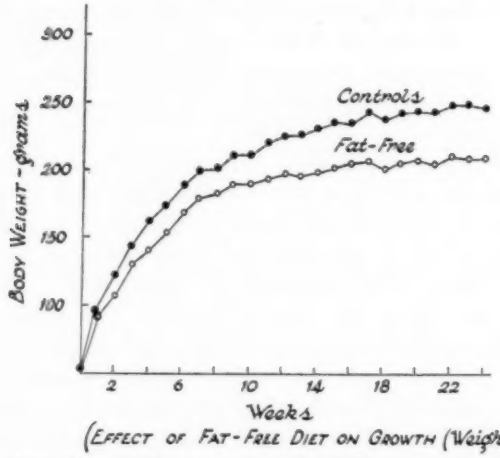


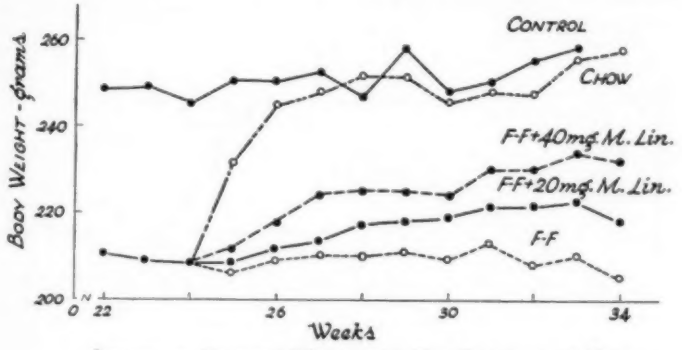

Fig. 1 Comparison of the curve of mean weight gain of 93 female rats on a fat-free diet for 24 weeks with that of control rats fed laboratory chow (Purina).

transferred to the chow diet after 24 weeks attained control weights within 4 weeks, after which they were indistinguishable from the controls. Animals receiving the methyl linoleate supplements experienced only partial recovery, which was more complete in those receiving 40 mg daily (fig. 2).

### *Appearance of skin*

Dermatitis, the characteristics of which have been previously described, began to appear after 9 weeks. The tail

showed involvement first, the hind feet two to three weeks later and the fore feet 5 to 6 weeks later. Seven animals died before the 10th week (autopsies revealed multiple pulmonary abscesses); the remaining 93 all developed dermatitis of the tail and feet as well as loss of hair, especially over the back. No dandruff was observed. Complete reversal of these changes occurred within 4 weeks in the groups transferred to the chow




EFFECT OF DIET ON WEIGHT OF FAT DEFICIENT ♀ RATS

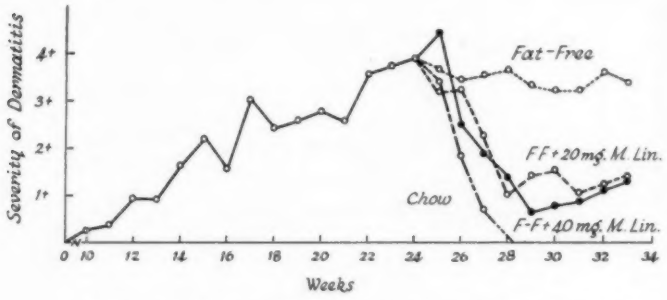
Fig. 2 Comparison of the curves of mean weight gain of fat-deficient rats with and without supplementation of the diet with methyl linoleate or laboratory chow. After 24 weeks on the fat-free diet, rats were separated into 4 dietary groups for an additional 10 weeks: fat-free diet; fat-free diet plus 20 mg methyl linoleate per day; fat-free diet plus 40 mg methyl linoleate per day; and laboratory chow diet.

diet. Marked but less complete recovery occurred in the groups receiving the methyl linoleate supplements; there was no apparent difference in degree of recovery in the two groups. Figure 3 depicts the course of the skin changes throughout the experiment; the ordinate expresses the mean weekly gradation of severity of dermatitis, in terms of criteria already described. There was no characteristic order of recovery in the various areas of involvement.

### *Ovulation*

The time of vaginal opening was not affected. There was a distinct tendency for irregularity of estrus in approximately

60% of the fat-free animals, most of which exhibited regular cycles for the first 20 weeks of the experiment and irregular cycles thereafter. The irregularity was characterized chiefly by variable prolongation of the cycle, and frequently by runs of diestrus. The addition of 20 mg methyl linoleate daily to the fat-free ration had no perceptible effect; that of 40 mg daily resulted in little improvement. Cycles were regular in all animals receiving chow.




EFFECT OF DIET ON SKIN MANIFESTATIONS OF FAT DEFICIENCY

Fig. 3 Graphic comparison of the severity of dermatitis in rats on a fat-free diet with the skin manifestations after addition of methyl linoleate or laboratory chow to the diet. Criteria for quantitative estimation are described in the text.

### *Oxygen consumption and blood pressure*

Data on oxygen consumption are presented in table 1. They indicate that the fat-deficient rats and those receiving 20 mg methyl linoleate daily utilized approximately twice as much oxygen as the controls. Of the group receiving 40 mg methyl linoleate daily, only two were tested: one had severe dermatitis and consumed as much oxygen as the fat-deficient animals; the skin of the other had undergone marked improvement, and its oxygen consumption was within the determined range for the control animals.

Because of the relative enlargement of the heart, blood pressures were recorded in a small number (6) of fat-

deficient animals in order to evaluate the possible role of hypertension in the production of this enlargement.[9] No abnormality in blood pressure was found.

TABLE 1

*Effect of diet on oxygen consumption*

| RAT NO. | TREATMENT | OXYGEN CONSUMPTION |
|---|---|---|
| | | *ml $O_2$/hr./100 gm* |
| 140 | Control | 88.6 |
| 144 | Control | 96.4 |
| 7 | Fat-free diet (34 weeks) | 135.0 |
| 16 (1) | Fat-free diet (34 weeks) | 159.0 |
| 16 (2) | Fat-free diet (34 weeks) | 143.0 |
| 34 | Fat-free diet (34 weeks) | 145.0 |
| 49 | Fat-free diet (34 weeks) | 220.0 |
| 26 (1) | Fat-free diet (34 weeks) plus 20 mg methyl linoleate/day during the last 10 weeks | 121.0 |
| 26 (2) | Fat-free diet (34 weeks) plus 20 mg methyl linoleate/day during the last 10 weeks | 124.0 |
| 62 | Fat-free diet (34 weeks) plus 20 mg methyl linoleate/day during the last 10 weeks | 159.0 |
| 87 | Fat-free diet (34 weeks) plus 40 mg methyl linoleate/day during the last 10 weeks | 131.0 |
| 65 | Fat-free diet (34 weeks) plus 40 mg methyl linoleate/day during the last 10 weeks | 94.6 |
| 52 | Fat-free diet (24 weeks) followed by control diet for 10 weeks | 95.5 |
| 68 | Fat-free diet (24 weeks) followed by control diet for 10 weeks | 84.7 |

[9] We wish to thank Dr. C. E. Hall and Dr. O. Hall, of the Department of Physiology, University of Texas Medical Branch, for making the measurements of blood pressure. The tail plethysmographic method of Williams, Harrison and Grollman ('39), as modified by Sobin ('46), was used. In each instance, the average of 4 successive readings which agreed to within 5 mm Hg was taken as representative of the blood pressure of the animal. In none of the 6 rats thus tested did the blood pressure approach 150 mm Hg, which is the arbitrarily established lower limit of hypertension used by these workers.

### *Macroscopic autopsy findings*

Table 2 depicts the organ weights of the animals sacrificed after 24 weeks. In terms of absolute weight, it will be seen that, in the fat-free animals, the pituitary, thyroid, thymus, adrenals, ovaries and uterus were smaller and the kidney larger than in the control animals. When computed on the basis of organ weight per 100 gm body weight, the thyroid and adrenals were still significantly smaller, whereas the kidneys, liver and heart were larger in the fat-free group than in the controls. The relative enlargement of the kidneys was particularly pronounced, being 43% larger in animals which were 20% smaller than the controls. Except for these weight differences, there were no perceptible changes on gross inspection of the specimens.

In studying the organ weights obtained at sacrifice after 34 weeks (i.e., at the termination of the experiment), it should be pointed out that the animals maintained on the fat-free diet became the group to which the changes in the other groups are to be compared. From table 2, it can be seen that in the animals transferred to the chow diet, the body weight and the weights of the pituitary, thyroid, thymus, adrenals, ovaries and uterus were significantly greater than in the fat-free group. When the weights were calculated on the basis of milligrams per 100 gm body weight, the most striking finding was the progressive relative decrease in the weights of the kidneys, liver and heart accompanying progressive improvement in nutrition. Thus, although there was a 25% increase in body weight in animals transferred to chow, there was a 20 to 25% decrease in the relative weights of these three organs. Again, gross inspection of all the organs revealed no differences other than in size and weight.

### *Microscopic studies*

Microscopic examination of the tissues of the various groups brought to light a number of significant findings. These were particularly striking in the case of the skin and kidneys of

TABLE 2

*Effect of a fat-free diet on weight of various organs of the growing female rat*

| DIET | NO. OF RATS | BODY WT. | PITUITARY WT. | THYROID WT. | THYMUS WT. | ADRENAL WT. | OVARY WT. | UTERUS WT. | KIDNEY WT. | LIVER WT. | HEART WT. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *gm* | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* |
| Control (24 weeks) | 10 | 245 ± 6[1] | 12.0 ± 0.4 | 17.1 ± 0.7 | 205 ± 12 | 40.2 ± 1.5 | 65.6 ± 3.4 | 478 ± 36 | 1,722 ± 43 | 7,868 ± 187 | 864 ± 30 |
| | | | (4.9)[2] | (7.0) | (85) | (20.2) | (28.0) | (194) | (704) | (3,212) | (354) |
| Fat-free (24 weeks) | 30 | 208 ± 2 | 9.1 ± 0.3 | 11.3 ± 0.3 | 160 ± 8 | 32.9 ± 0.7 | 52.9 ± 1.9 | 357 ± 21 | 2,082 ± 42 | 8,174 ± 200 | 873 ± 17 |
| | | | (4.4) | (5.4) | (77) | (15.9) | (25.6) | (178) | (1,003) | (3,930) | (420) |
| Fat-free (34 weeks) | 14 | 205 ± 5 | 9.2 ± 0.4 | 11.9 ± 0.4 | 117 ± 9 | 33.0 ± 0.6 | 52.8 ± 2.8 | 329 ± 30 | 2,004 ± 61 | 8,153 ± 277 | 759 ± 23 |
| | | | (4.5) | (5.8) | (58) | (16.0) | (25.8) | (159) | (979) | (4,021) | (370) |
| Fat-free (34 weeks) plus 20 mg meth. lin. daily last 10 weeks | 12 | 217 ± 5 | 9.3 ± 0.3 | 12.5 ± 0.4 | 164 ± 13 | 33.8 ± 1.1 | 55.4 ± 2.9 | 463 ± 37 | 1,801 ± 65 | 7,510 ± 211 | 821 ± 26 |
| | | | (4.2) | (5.8) | (75) | (15.7) | (25.7) | (217) | (834) | (3,486) | (379) |
| Fat-free (34 weeks) plus 40 mg meth. lin. daily last 10 weeks | 8 | 232 ± 1 | 10.9 ± 0.7 | 13.1 ± 0.3 | 161 ± 13 | 32.5 ± 2.3 | 54.3 ± 4.0 | 473 ± 50 | 1,930 ± 73 | 7,614 ± 280 | 753 ± 16 |
| | | | (4.7) | (5.6) | (70) | (14.0) | (23.4) | (203) | (831) | (3,149) | (324) |
| Fat-free (24 weeks) Chow (10 weeks) | 11 | 257 ± 5 | 12.3 ± 0.5 | 15.4 ± 0.9 | 173 ± 2 | 41.5 ± 1.4 | 64.3 ± 2.1 | 502 ± 15 | 1,887 ± 42 | 7,834 ± 258 | 757 ± 26 |
| | | | (4.8) | (6.0) | (67) | (16.2) | (25.0) | (196) | (736) | (3,047) | (295) |

[1] Standard error.

[2] Relative weight (mg/100 gm body weight).

the fat-deficient animals, and somewhat less striking with respect to the pituitary, ovaries and liver. There were no significant histologic alterations in the thyroid, thymus, adrenals, pancreas, uterus or heart.

*Skin.* Skin changes were most marked and occurred in every fat-free animal. Specimens taken from both dorsal and ventral areas revealed the same findings, the corium being thicker in the former. In the normal rat (figs. 4 and 6, plate 1) the epidermis was thin (one to two cell layers deep). The stratum corneum was thin and lacy in appearance. There was no differentiation into cell layers, although there was a thin layer of keratohyalin granules. The cell outlines were indistinct and the cytoplasm scanty; the nuclei were compact and contained well-dispersed chromatin. In the fat-deficient rat (figs. 5 and 7, plate 1), the epidermis was greatly thickened (6 to 12 cell layers deep) and there was greater differentiation into layers. The stratum corneum was markedly hyperkeratotic and occasionally thicker than the whole epidermis; the keratin was disposed in thick sheets; parakeratosis (presence of nuclei in stratum corneum) was infrequently observed. The stratum granulosum was well-developed, consisting of several layers and containing large amounts of deeply staining dispersed keratohyalin granules; the cytoplasm frequently appeared vacuolated. The cell outlines were very distinct, the intercellular bridges frequently discernible; the cytoplasm was abundant, making the cells appear plump; the nuclei appeared somewhat larger and there was a tendency toward clumping of the chromatin. In the corium, the collagen appeared edematous and disorganized and there was more cellular infiltration than in the control. The epithelium of the hair follicles was markedly hypertrophied and, in some areas, the hairs seemed to be broken off in the follicle. There was some epidermal plugging observed but it was not extensive.

Essentially the same changes were present in the skin of animals which received the 20-mg methyl linoleate daily supplement for 10 weeks, except for a definite reduction in the number of cell layers, from 8 to 12 to two to 4. Skin from

animals of the 40-mg group revealed a considerable improvement but hyperkeratosis and plump cells persisted even in those instances in which the skin, to gross inspection, appeared entirely normal. There was likewise a distinct tendency toward hyperkeratosis in specimens from animals transferred to the chow diet, in which the skin had appeared normal for an average period of 6 weeks before sacrifice.

*Kidneys.* In the fat-free group the outstanding and characteristic finding was the large accumulation of blood between tubules, particularly between the ascending and descending loops of Henle. Frequently, endothelial cells could be discerned lining these accumulations. In many sections, the glomerular tufts appeared large and congested, with reduction in or obliteration of the usually observed capsular space. The brush border of the proximal tubules was definitely wider, or thicker, than in control specimens. There was a variable amount of intra-tubular debris, but in no instance were erythrocytes or granular hyaline bodies observed within the tubules (figs. 8 and 9, plate 2).

The findings in animals receiving 20 mg methyl linoleate daily were essentially the same, but were somewhat less pronounced. In those receiving daily 40 mg, the capillary hyperemia persisted, but to a much lesser degree. Sections of the chow group differed little from the controls, except for occasional evidences of capillary hyperemia.

*Ovaries.* The follicles and corpora lutea of ovaries from the fat-deficient animals appeared normal on microscopic examination, although there was a tendency for the follicles to be larger than in the controls. The most striking finding was the great number of "wheel nuclei" in cells derived from the spindle cells of the persistent thecae internae of atretic follicles, as originally described by Selye and co-workers ('33a, b), and said to represent deficiency of pituitary secretion of luteinizing hormone (figs. 10 and 11, plate 2). Most ovaries of the fat-free group contained a larger amount of interstitial tissue than those of the other groups.

*Liver.* No significant differences were observed in sections stained with hematoxylin-eosin. Sections stained with Sudan IV revealed minimum deposits of fat, particularly near the center of the lobules but also in the periportal areas in most of the fat-free animals.

*Pituitary.* Fat deficiency for 24 weeks resulted in a marked reduction in the percentage of acidophile cells: controls 33.4%; fat-free 21.1%. The percentage of basophiles was increased

TABLE 3

*Effect of a fat-free diet on the cytological structure of the hypophysis of growing female rats*

| TREATMENT | NO. OF RATS | BODY WT. | PITUITARY CELL ANALYSIS | | |
|---|---|---|---|---|---|
| | | | Acidophiles | Basophiles | Chromophobes |
| | | *gm* | % | % | % |
| Control | 7 | 242 ± 6[1] | 33.4 ± 0.9 | 4.9 ± 0.4 | 61.7 ± 1.1 |
| Fat-free, 24 weeks | 10 | 206 ± 5[2] | 21.1 ± 1.2[2] | 9.4 ± 1.5[2] | 69.5 ± 1.3[2] |
| Fat-free, 34 weeks | 8 | 210 ± 6[2] | 21.2 ± 3.1[2] | 7.5 ± 0.5[2] | 71.3 ± 3.3 |
| Fat-free, 34 weeks plus 20 mg methyl linoleate daily for last 10 weeks | 10 | 222 ± 6 | 22.8 ± 1.0[2] | 6.5 ± 0.4 | 70.7 ± 1.4[2] |
| Fat-free, 34 weeks plus 40 mg methyl linoleate daily for last 10 weeks | 7 | 235 ± 2 | 18.8 ± 3.2[2] | 6.1 ± 0.6 | 75.1 ± 3.1[2] |
| Fat-free, 24 weeks Chow 10 weeks | 10 | 256 ± 6 | 25.9 ± 2.2[2] | 6.7 ± 0.8 | 67.4 ± 1.8 |

[1] Standard error.

[2] Statistically significant difference from control value.

to 9.4% as compared to a control value of 4.9%. Increased size of basophile cells was apparent in the fat-free rats, as well. Similar results obtained after 34 weeks of fat deficiency (table 3). Addition of methyl linoleate to the diet had no effect on acidophile percentage but a tendency toward a normal percentage of basophiles was found. The rats transferred to chow showed an intermediate cellular content, between that of the controls and the fat-deficient animals.

## DISCUSSION

The fat-deficiency signs produced in this experiment compare closely with those described in numerous previous reports. The large number of animals employed (100) and the duration of the experiment (34 weeks) imposed rigid test conditions for the use of a synthetic, fat-free diet.

The impairment of growth was apparent earlier than in many studies; i.e., after two weeks as contrasted with after 60 days (Evans and Burr, '27a; Maeder, '37; Hume et al., '38). A growth plateau was reached after 112 to 119 days, a common finding (Burr and Burr, '29; Maeder, '37; Turpeinen, '38; Martin, '39), although others have reported the plateau to occur after 60 to 77 days (Evans and Lepkovsky, '32; Hume et al., '38; Greenberg et al., '50). The average weight at the plateau was 200 gm. As in other reports dealing with females, cessation of weight gain occurred when the weight was approximately 80% of that of the controls (Burr and Burr, '29; Maeder, '37).

The histologic changes in the skin were similar to those in the original description by Williamson ('41) in rats, and essentially identical to those in fat-deficient dogs described by Hansen, Holmes and Wiese ('51).

Hematuria was early emphasized as an important finding by Burr and Burr ('29, '30) and was stated to be a measure of the severity of fat deficiency. Other workers confirmed this, to a rather variable degree (Evans and Lepkovsky, '32; Maeder, '37; Hume et al., '38; Turpeinen, '38; Martin, '39). Burr ('42) later reported that hematuria occurs late and without regularity (16 to 90%), although "kidney lesions" occur in 100% of fat-free animals. In the present study, hematuria was not observed in any of the fat-free animals, although microscopic pathologic findings were present in all kidneys. The only previous work known to the authors reporting histologic studies of the kidneys of fat-deficient animals is that of Borland and Jackson ('31), which presents findings widely divergent from those in this presentation. The most striking lesions described in their report were calcium deposits within

cortical tubular cells and lumina of the tubules; there was also much simple degeneration of the tubular cells. No attempt was made to correlate any of their findings with hematuria. Except for the thickening of the brush border of proximal tubules, no abnormality of tubular structure was found in the present study. The most consistent and striking characteristic was the great accumulation of red cells between the tubules; in no instance was there evidence of blood within the lumen of the tubules (or in the bladder).

The tendency for irregular ovulation observed in this experiment is in agreement with other studies. Evans and Burr ('27a, '28) reported that ovulation did not occur at all in the first 4 months, after which the rate of ovulation was less than 50% of that of control animals. Burr and Burr ('29, '30) state that approximately 70% of fat-free animals ovulate irregularly, that severe fat deficiency may be produced before ovulation is affected and that many animals cease ovulating completely. Rather severe disturbance of estrous cycles was observed by Turpeinen ('38) among a group of 82 fat-deficient animals, over 90% showing some irregularity, including a few which ceased to ovulate altogether. Maeder ('37) studied 57 fat-deficient animals and reported that only 20% had irregular estrous cycles. In the present study, ovulation was regular for 18 to 20 weeks, after which it became irregular in 60% of the animals; complete cessation of estrous cycles was not observed. The effect of fat deficiency on estrous cycles would seem, therefore, to be definite, insidious in onset and not profound (see below for remarks regarding a possible mechanism).

Perhaps the most striking effect of fat deficiency herein reported is that on the liver, heart and kidneys. As has been emphasized, these organs undergo relative enlargement during the development of the syndrome, and in the case of the kidneys, an absolute increase in weight as well. Adequate explanation for these changes is not possible on the basis of the information available. On the basis of microscopic study, it seems likely that the intertubular accumulations of

blood account for most, if not all, of the kidney enlargement. There were no perceptible changes in sections of the heart, although fiber size was estimated but not measured; there was suggestive evidence of increased capillary blood supply, but this could hardly be responsible for the weight change. Similarly, the minimum amounts of fat noted in the specially stained sections of the liver could not explain the increase in liver size.

Evidence is accumulating, however, that these three organs are particularly involved in fatty acid metabolism. Spectrophotometric determinations of tissue fatty acid content reveal that normal rats fed a stock ration show the presence of large amounts of polyunsaturated fatty acids in the heart, liver, brain, kidney, blood and skeletal muscle, in decreasing order of concentration (Widmer and Holman, '50). Fat-deficient rats fed a good source of unsaturated fatty acids, such as cod liver oil or single essential fatty acids, linoleic or arachidonic, show strikingly large depositions of fatty acids in the heart, liver, brain and kidney (Rieckehoff, Holman and Burr, '49; Widmer and Holman, '50; Holman and Taylor, '50). The response of tissue fatty acid content to dietary change is sensitive more or less in the same order in which organs retain highly unsaturated fatty acids when fat-starved for long periods; this suggests that heart, liver, brain and kidney require highly unsaturated acids, particularly the heart, the fatty acid content of which varies more sensitively to dietary change than that of any other organ (Rieckehoff et al., '49). It would seem more than coincidental, therefore, that the organs which show the greatest fatty acid metabolic activity are those which undergo the greatest change in weight in fat-free animals. Such biochemical data provide no explanation for the changes in weight, however, and further study will be necessary to elucidate this apparent correlation.

The identification of large numbers of "wheel cells" in the interstitial tissue of the ovaries of fat-deficient animals is a finding of some significance. These cells regularly ap-

pear following hypophysectomy in the rat and disappear following the injection of crude or purified preparations of luteinizing hormone (Selye, Collip and Thomson, '33a, b; Evans and Simpson, '50). Their appearance is considered to be an exceedingly sensitive indicator of gonadotrophic insufficiency, since the ovaries of immature rats show the development of such cells following hypophysectomy; normally, the gonadotrophic output of the pituitary of immature rats is insufficient to induce the formation of mature follicles and corpora lutea, but apparently is capable of preventing the appearance of "deficiency cells" (Selye, '33; Collip, Selye and Williamson, '38). On the basis of this evidence, it is reasonable to assume that the appearance of "wheel cells" in the ovaries of fat-deficient rats is indicative of a diminished production of luteinizing hormone by the pituitary.

That impaired secretion of gonadotrophin may occur in fat deficiency is suggested by reports of experiments designed to impose a burden on the pituitary-ovarian system. Successful gestation or lactation cannot be achieved in fat-deficient female rats; depending on the severity of signs of deficiency, failure of implantation, early resorption or prolonged gestation occur (Evans, Lepkovsky, and Murphy, '34a, b, c; Maeder, '37). Almost identical results can be produced by hypophysectomy in pregnant rats (Selye, Collip and Thomson, '33c; Pencharz and Long, '33). The exceptional similarity of such findings supports the speculation that similar etiologic mechanisms are involved.

Fat-deficient males exhibit subnormal sex interest, usually do not copulate at all and are infertile when copulation does occasionally take place (Burr and Burr, '30; Evans, Lepkovsky and Murphy, '34c). Curative doses of fatty acid prepared from corn oil lead to rapid restoration of sex interest and gradual return of fertility in the majority of instances (Evans et al., '34c). These abnormalities can be attributed to failure of synthesis of testicular hormone or to "grave degeneration of the testes," as suggested by the two groups of authors, respectively. A possible alternative explanation

would be that fat deficiency causes impairment of pituitary function and that the abnormalities could be due to inadequate or disturbed gonadotrophic stimulation of the testes.

The marked fall in the number of pituitary acidophiles and the small but significant rise in basophiles provide additional evidence which, when considered in the light of the disturbed ovulation and the observed "deficiency cells" of the ovary, permit the conclusion that an important effect of fat deficiency is a decrease in the pituitary secretion of luteinizing hormone (LH). The correlations which seem to justify this statement are the following: (1) experimental work has identified pituitary acidophile activity with the production of LH (Severinghaus, '37, '39; Dawson, '46; Meyer, Biddulph and Finerty, '46); (2) proportional shifts in pituitary cell types, occurring in conjunction with physiologic changes, are useful indicators of activity (Severinghaus, '39); a decrease in number of acidophiles, as reported in this experiment, may therefore be indicative of diminished production of LH; (3) the appearance of "wheel cells" in the ovarian interstitial tissue has been closely correlated with deficiency of LH (Selye, Collip and Thomson, '33a, b; Evans and Simpson, '50); the finding of such cells was conspicuous in the present study and may be interpreted as additional evidence for decreased secretion of LH; (4) reduced secretion of estrogen by the ovary is to be expected when that gland is inadequately stimulated by LH, since it is known that the latter hormone is necessary for activating the follicles to produce estrogen (Evans and Simpson, '50); the impaired ovulation noted in this experiment is compatible with decreased estrogen secretion; (5) the so-called "castration effect" of increase in pituitary basophiles associated with decrease in or absence of estrogen production is well-known (Severinghaus, '39; Meyer et al., '46); with the evidence heretofore discussed in this presentation, it seems logical to attribute the observed rise in number of basophiles in the pituitaries of fat-free rats to the decreased secretion of estrogen.

## SUMMARY AND CONCLUSIONS

Characteristic signs of fat deficiency were produced in 93 female rats on a fat-free diet for 34 weeks from the time of weaning. Data from this study show that:

1. The maximum weight attained by fat-deficient rats was 80% that of controls. When computed on the basis of organ weight per 100 gm of body weight, the thyroid and adrenal glands were significantly smaller and the kidneys, liver and heart larger in the fat-free group than in the controls. The kidneys were heavier on an absolute weight basis as well. Addition of fat to the experimental diet resulted in rapid increase in body weight to control values and progressive relative decrease in weight of kidneys, liver and heart. Microscopic examination of the kidneys revealed large inter-tubular accumulations of blood but no intra-tubular findings.

2. All experimental animals developed dermatitis of the tail and feet and loss of hair over the back. Histologic alterations were pronounced in the epidermis and dermis.

3. Estrous cycles became distinctly irregular after 18 to 20 weeks of the fat-free diet. Ovarian weight was not affected, but large numbers of "deficiency" nuclei were present in the interstitial tissue. Follicles and corpora lutea appeared normal. Quantitative analysis of pituitary cell types revealed a significant decrease in percentage of acidophiles and an increase in basophiles. These findings suggest that fat deficiency causes a reduced secretion of luteinizing hormone from acidophile cells and that the consequent reduction in ovarian stimulation effects a diminished estrogen secretion with consequent disturbance of estrous cycles. Increased percentage of basophiles (castration changes) can be attributed to reduced secretion of estrogen.

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# PLATES

## PLATE 1

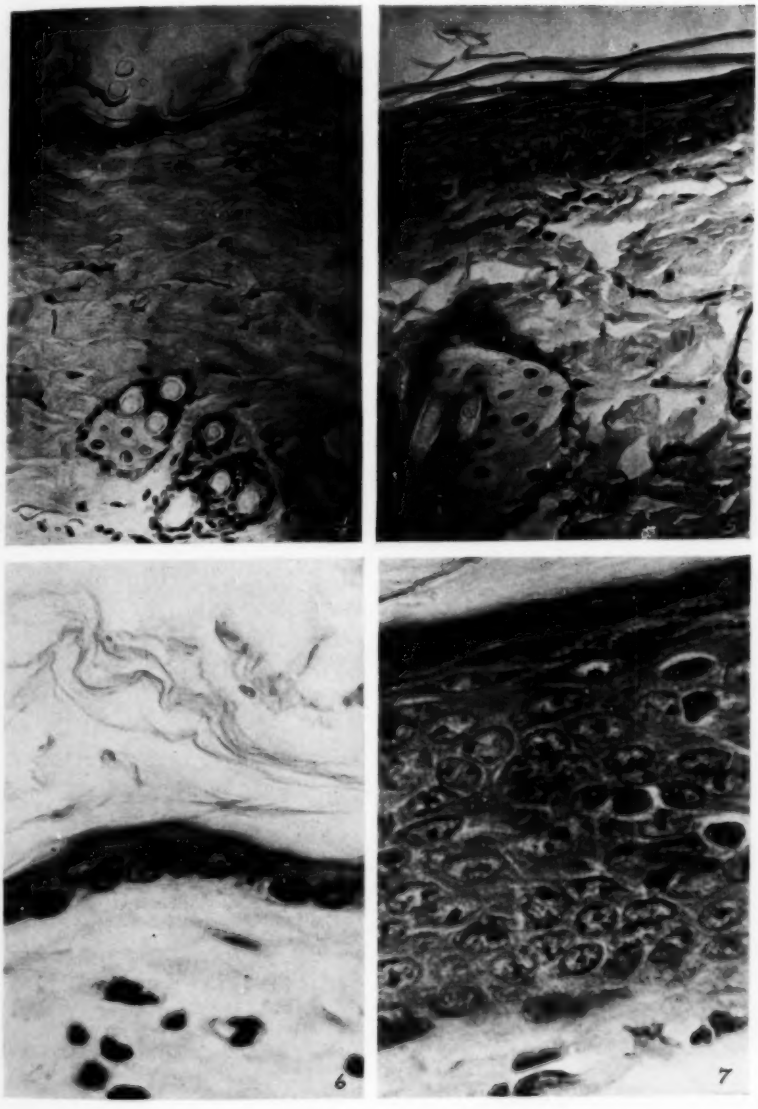
### EXPLANATION OF FIGURES

4 Microscopic section of interscapular skin from a control rat (H and E stain; 300 ×).

5 Microscopic section of interscapular skin from a rat on a fat-free diet for 24 weeks. Note plate-like thickening of the stratum corneum; increased deposition of granules and number of layers of the stratum granulosum; and proliferation of stratum germinativum. Changes in the dermis consist of irregularity and disruption of collagenous bundles; increase in number of cells; and abnormal hypertrophy of hair follicles and sebaceous glands (H and E stain; 300 ×).

6 Higher magnification of normal interscapular skin (H and E stain; 975 ×).

7 Higher magnification of interscapular skin from a fat-free rat. Note the unusual development of stratum granulosum, and increased thickening of stratum germinativum with prominent intercellular bridges (H and E stain; 975 ×).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## PLATE 2

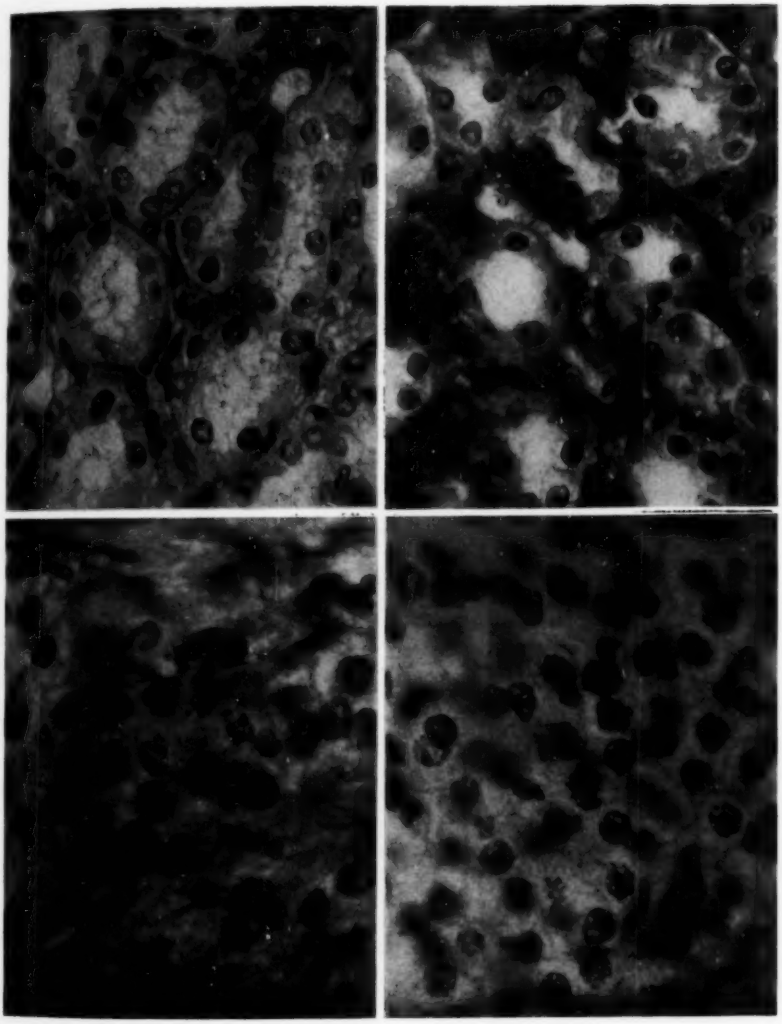
### EXPLANATION OF FIGURES

8 Microscopic section of uriniferous tubules in the renal cortex of a control rat (H and E stain; 599 ×).

9 Microscopic section of uriniferous tubules in the renal cortex of a rat on a fat-free diet for 24 weeks (H and E stain; 599 ×). Note accumulations of red cells between tubules and the absence of debris in the tubular lumen.

10 Microscopic section of ovarian interstitial tissue of a control rat (H and E stain; 931 ×).

11 Microscopic section of ovarian interstitial tissue of a fat-deficient rat (H and E stain; 931 ×). The presence of "wheel nuclei" is conspicuous.



# LEUKOCYTE PRODUCTION IN PANTOTHENIC ACID-DEFICIENT MICE [1]

DAVID R. WEIR

*Department of Medicine, School of Medicine, Western Reserve University and University Hospitals, Cleveland, Ohio*

FOUR FIGURES

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Study of the roles of hormones and vitamins in the formation of leukocytes in mice has shown that cortisone treatment and pyridoxine deficiency produce granulocytosis, lymphopenia, and myeloid metaplasia in the spleen (Weir, Heinle and Welch, '49; Weir and Heinle, '50). Similar granulocytosis and lymphopenia were reported to occur in pantothenic acid-deficient monkeys (McCall, Waisman, Elvehjem and Jones, '46). Hoping to compare the hematologic effect of deficiency of this vitamin with our previous findings, attempts were made to establish pantothenic acid deficiency in mice. Preliminary findings indicated that granulocytosis and myeloid metaplasia resulted, but lymphopenia was not observed. This combination of findings also occurs when mice are treated with a myeloid metaplasia factor of human urine (Weir and Heinle, '50) and, temporarily, when adrenalectomized mice are treated with adrenocorticotropic hormone, as previously reported (Dougherty and White, '44) and confirmed in this laboratory (fig. 1). Based on these preliminary observations, further efforts were made to produce pantothenic acid deficiency in mice.

[1] These studies were supported by a grant from the National Vitamin Foundation.

## METHODS

Various antagonists of pantothenic acid have been used in attempts to facilitate the production of pantothenic acid deficiency (Moss, Urist and Martin, '48; Drell and Dunn, '48; Shils, '50; Drell and Dunn, '51). Using weanling mice of a non-leukemic Swiss strain that have been bred in this laboratory for 9 years, various combinations of diet and antagonist were used. It was hoped that the deficiency could be produced by giving large amounts of antagonist while maintaining the animals on a normal diet rather than on a deficient

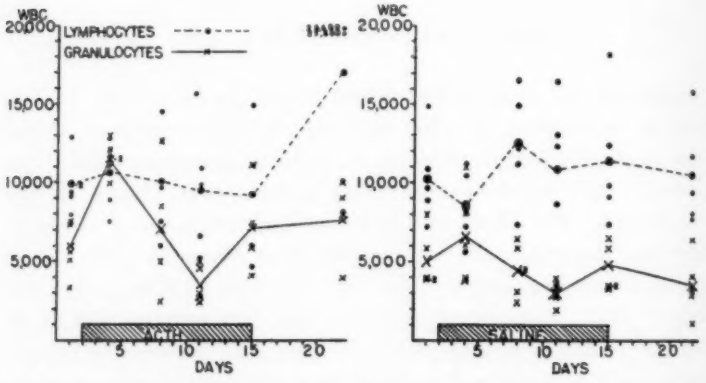



Fig. 1 Leukocyte changes in adrenalectomized mice. The dose of ACTH was 2.5 mg intraperitoneally every 8 hours.

diet. In computing the ratios of antagonist to vitamin the commercial dog food [2] used was assumed to contain 6.65 p.p.m. of pantothenic acid, as reported by the manufacturer. In preliminary experiments the regimens shown in table 1 were used; there were 5 mice in each experiment.

As will be discussed below, none of these regimens regularly produced pantothenic acid deficiency. In addition there was no evidence that the use of the antagonists gave any advantage over the use of the deficient diet alone.

[2] Purina dog chow.

Taking into account the report (Lichstein and Gilfillan, '51) that streptomycin inhibits the synthesis of pantothenate from beta-alanine and pantoyl lactone, a final experiment was devised using a deficient diet (King, Strong and Cheldelin, '50)

TABLE 1

*Antagonists of pantothenic acid used in attempts to facilitate production of pantothenic acid deficiency*

| ANTAGONIST | DIET | RATIO OF ANTAGONIST TO VITAMIN | % ANTAGONIST | DURATION |
|---|---|---|---|---|
| | | | | *days* |
| Salicyloyl-beta-alanide | Dog chow | 100: 1 | | 1–8 |
| | | 200: 1 | | 9–25 |
| | | 400: 1 | | 26–45 |
| | | 800: 1 | | 46–57 |
| Pantoyltaurine | Dog chow | 50: 1 | | 1–24 |
| | | 100: 1 | | 25–35 |
| | | 200: 1 | | 36–75 |
| Pantoyltaurine | Dog chow | 400: 1 | | 1–56 |
| | | 800: 1 | | 57–112 |
| Omega-methyl-pantothenic acid | Dog chow | 200: 1 | | 1–63 |
| | | 400: 1 | | 64–112 |
| None | Pantothenic-deficient | | | 68 |
| Pantoyltaurine | Pantothenic-deficient | | 0.14 | 77 |
| Pantoyltaurine | Pantothenic-deficient | | 0.56 | 162 |
| Omega-methyl-pantothenic acid | Pantothenic-deficient | | 0.14 | 25 |

without an antagonist. Ten weanling Swiss mice approximately 21 days old and weighing 10 to 15.4 gm were divided into two groups of 5 each and paired as closely as possible according to weight. The animals were pair-fed, the control animal of each pair being fed the amount of food eaten by the corresponding experimental animal on the previous day. The animals were weighed daily and it was planned to starve

the control animals, if necessary, to maintain their weights equal to those of the experimental animals. This was not necessary, as the paired feeding alone accomplished the desired result. All animals were fed the pantothenic acid-deficient diet to which was added 0.17% streptomycin. In addition, 0.075% calcium pantothenate was added to the diet of the control animals. White blood cell counts and differential counts were done on all animals twice weekly. At autopsy sections of the liver, spleen, bone marrow, adrenals and lymph nodes were taken for histologic study.

## RESULTS

For the final experiment, in which streptomycin was used in conjunction with a deficient diet without an antagonist, the changes in the granulocyte counts are shown in figure 2. A moderate but definite rise occurred in the counts for the experimental animals, while those of the control animals remained at normal levels. Lymphopenia did not occur. In fact, the lymphocyte counts in both the experimental and control animals rose moderately from the initial counts, but not to levels which are abnormal for this strain of mice. Weight gain was slow at first and finally ceased at subnormal levels (fig. 3). After the 24th day none of the pantothenic-deficient animals or their pair-fed controls grew at a rate comparable to that of any of 5 animals of the same strain fed dog chow ad libitum. No other obvious signs of pantothenic acid deficiency were observed.

In the preliminary experiments, deficiency of pantothenic acid was obtained in some of the experimental animals, but this was overcome spontaneously with resumption of normal growth. Not only did some animals fail to develop the deficiency, but there was great variation in the susceptibility of individual animals to the development of deficiency. Under identical conditions one animal might show only temporary granulocytosis and arrest in weight gain, while another would develop obvious pantothenic acid deficiency. The signs of this, which included steady weight loss or failure to gain,

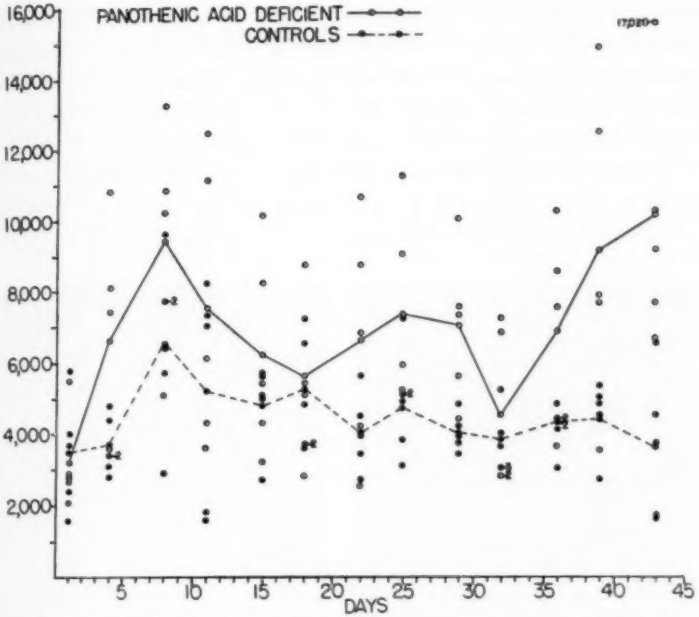



Fig. 2 Circulating granulocyte counts in pantothenic acid deficiency.

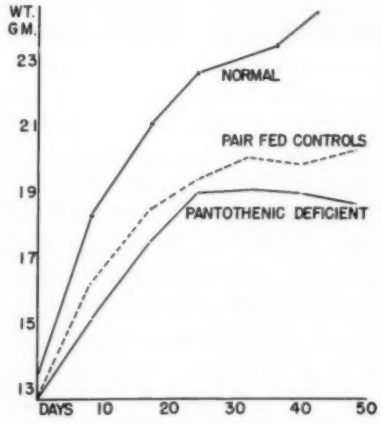
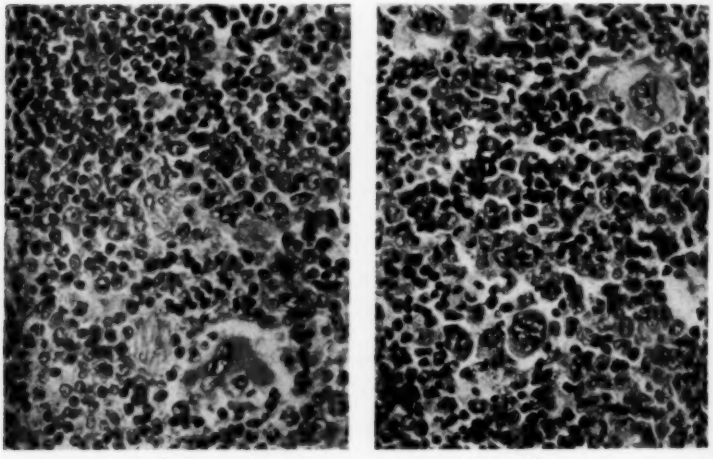



Fig. 3 Arrest of growth in pantothenic acid deficiency.

ruffled fur, dermatitis, partial paralysis of extremities and granulocytosis without lymphopenia, could all be corrected promptly by the addition of calcium pantothenate to the diet. The granulocytosis without lymphopenia routinely accompanied the other signs of deficiency.

A number of the animals that were obviously deficient died or were killed and subjected to autopsy. In some, but not all, of these, marked myeloid metaplasia occurred in the spleen



A B

Fig. 4 Spleens × 370.
(A) Normal control. (B) Pantothenic acid-deficient.

(fig. 4). The pulp was densely infiltrated with polymorphonuclear granulocytes, large immature mononuclear cells, and numerous mitoses. Detailed cytologic studies designed to determine the identity of the immature mononuclear cells and mitoses were not done. Inasmuch as these cells were found in the pulp of the spleen rather than in the centers of the follicles, and were in the company of numerous adult polymorphonuclear granulocytes, it was believed probable that they were cells of the granulocyte series corresponding

to human myelocytes and myeloblasts. Nucleated red blood cells and megakaryocytes were present in normal numbers in the splenic pulp. The lymph follicles of the spleen were somewhat hypoactive but by no means inactive, while the follicles of the lymph nodes taken from various locations in the body were normal in appearance. Thus there was histologic evidence as well as evidence in the peripheral blood to show that lymphopoiesis was not diminished.

The mouse bone marrow normally contains chiefly polymorphonuclear granulocytes, nucleated red cells, and megakaryocytes, with a few immature mononuclear cells. Also there is no fat, making it difficult to detect minor changes in its cellular composition. In the deficient animals significant changes in the marrow did not occur. The adrenals appeared normal, although, again, detailed cytologic studies were not done. The livers were normal.

## DISCUSSION

It is apparent that the production of pantothenic acid deficiency in the mouse is difficult, with considerable variation in individual susceptibility and in strain susceptibility (Fenton, Cowgill, Stone and Justice, '50). Also escape from deficiency occurs and has been explained (Fenton, Cowgill, Stone and Justice, '50) on the basis that young mice growing rapidly need larger amounts of pantothenic acid than do older mice, and hence are more easily depleted. Further evidence for this is to be found in the report (Lotspeich, '50) that adult rats maintained on a pantothenic acid-deficient diet are quickly made deficient in the vitamin if given anterior pituitary growth hormone. Other explanations for failure to develop the deficiency might be made. Acquisition by the animal of the ability to utilize the antagonist as a vitamin, or to by-pass its effect by a metabolic change, could occur. Intestinal synthesis could become an important source of vitamin.

In our experience we could not prove that any antagonist of pantothenic acid produced deficiency in our strain of mice

more quickly or completely than a deficient diet alone, although there were indications that omega-methylpantothenic acid might have some value in this respect.

In the animals that did become deficient, granulocytosis without lymphopenia occurred regularly and could be corrected or prevented by giving calcium pantothenate. We do not agree with Melampy, Cheng and Northrop ('51) that significant lymphopenia and insignificant neutrophilia occur. The granulocytosis without lymphopenia also occurs in adrenalectomized animals treated with ACTH and in mice treated with extracts of urine from patients having myeloid leukemia. In pyridoxine deficiency, cortisone treatment and treatment with ACTH in intact animals, the granulocytosis is accompanied by lymphopenia. The significance of the lack of lymphopenia in pantothenic acid deficiency is not clear at the present time. That pantothenic acid may have some role in the control of the production of granulocytes may be surmised. Also it seems possible that this control is not dependent upon the usual reciprocal relationship between granulocytes and lymphocytes. Some other factor may be operative and may also be operative in myeloid leukemia of human beings, as indicated by the fact that the urine extracts of these patients produce changes similar to those of pantothenic acid deficiency; namely, granulocytosis without lymphopenia and myeloid metaplasia in the spleen.

We cannot draw any conclusions about the possible value of streptomycin in helping to produce pantothenic acid deficiency in mice. Further controlled experiments are necessary.

## SUMMARY

1. In pantothenic acid-deficient mice granulocytosis without lymphopenia occurs and is accompanied by myeloid metaplasia in the spleen.

2. In mice there is individual and strain variation in susceptibility to the establishment of pantothenic acid deficiency.

ACKNOWLEDGMENT

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# THE QUANTITATIVE THREONINE REQUIREMENT OF THE SUCKLING PIG

R. F. SEWELL, J. K. LOOSLI, L. A. MAYNARD, HAROLD H. WILLIAMS AND BEN E. SHEFFY

*Departments of Animal Husbandry, and Biochemistry and Nutrition, Cornell University, Ithaca, New York*

ONE FIGURE

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## INTRODUCTION

Previous studies on the amino acid requirements of swine have been carried out by supplementation of proteins that were low in one or more amino acids. This method has been used to study the quantitative requirements of weanling pigs for tryptophan (Shelton et al., '51a), lysine (Brinegar et al., '50b; Shelton et al., '51b), isoleucine (Brinegar et al., '50a) and methionine (Shelton et al., '51c). However, a paucity of proteins deficient in the remaining naturally occurring amino acids has limited evaluation of their significance in swine nutrition by this technique.

Shelton et al. ('50) used a purified diet which had as its sole sources of nitrogen diammonium citrate and the 10 amino acids shown to be essential for the growing rat (Rose, '38), to demonstrate that threonine is an indispensable dietary component for swine.

Using the same type of diet, Beeson et al. ('51) showed that the amino acids arginine, leucine, phenylalanine, and valine are also essential for optimum growth of the weanling pig.

The study described in the present report was designed to determine the quantitative threonine requirement of suckling

pigs, using a simulated milk diet in which the nitrogen was supplied by isolated soybean protein,[1] crystalline amino acids, and ammonium citrate (dibasic).

## PROCEDURE

Two- to three-day old Yorkshire pigs were placed on a semi-synthetic milk diet which was prepared as described by Clark ('27) and Kastelic et al. ('50) with some modifications. The pigs were fed a basal diet containing casein as a source of protein for a two-day period to adjust them to the method of bottle feeding which was to be used in the study. Individual battery-type cages were used to house the pigs. Each pig was fed individually three times a day from a bottle provided with a lamb's nipple so as to reduce feed wastage to a minimum. The feeding bottles were held in a rack especially designed for this purpose.

Following the two-day adjustment period on the casein diet, the pigs were placed on their respective experimental diets for an additional 7-day transitional period. The basal diet, as shown in table 1, contained isolated soybean protein which had been washed to remove the sulfites. This provided a portion of the essential amino acid nitrogen as well as some non-essential amino acids. Amino acid values for the washed alpha protein were determined by microbiological assay (Williams and Abraham, '52). The remainder of the essential amino acid nitrogen of the diet was supplied as the crystalline amino acids. Ammonium citrate (dibasic) provided the balance of the dietary nitrogen on a protein equivalent basis.

Threonine was added to the different diets at various levels so as to give 5 experimental diets of differing threonine content. The threonine added to each diet replaced an equivalent amount of glucose, giving a range in protein content for the 5 experimental diets of from 24.17 to 25.50%. Growth response and efficiency of feed utilization were used as criteria

[1] "Alpha" protein, Glidden Co., Chicago, Ill.

of measurement over a 21-day period. The pigs were fed all of the milk diet that they would consume.

## RESULTS

Rose ('38) has reported that only the naturally occurring isomer of threonine is useful in promoting growth in the rat. Nasset et al. ('51) have likewise shown that only the L-form is utilized for maintenance of nitrogen equilibrium in the adult rat. Assuming that the same is true for pigs, the

TABLE 1

*Ingredients and amino acid composition of the basal diet* [1]

| INGREDIENTS | AMOUNT | AMINO ACIDS | AMOUNT |
|---|---|---|---|
| | % | | % |
| Alpha protein [2] | 11.19 | Arginine | 1.20 |
| Amino acid mixture [3] | 4.74 | Histidine | 0.46 |
| Ammonium citrate (dibasic) | 11.85 | Isoleucine | 0.77 |
| Cerelose | 38.11 | Leucine | 1.44 |
| Lard | 24.63 | Lysine | 1.34 |
| Soya lecithin [4] | 1.49 | Methionine | 0.72 |
| Minerals [5] | 8.73 | Phenylalanine | 1.20 |
| Vitamins [6] | + | Threonine | 0.36 |
| | | Tryptophan | 0.24 |
| | | Valine | 1.01 |
| | | Tyrosine | 0.31 |

[1] Aureomycin, supplied by the Lederle Laboratories, Pearl River, New York, was added to all rations at the rate of 150 mg/kg D.M.

[2] Washed to remove the sulfites.

[3] Contained the following crystalline amino acids: L-arginine, 10.0%; L-histidine, 4.5%; DL-isoleucine, 10.3%; L-leucine, 13.4%; L-lysine, 16.5%; DL-methionine, 12.9%; DL-phenylalanine, 13.6%; DL-tryptophan, 3.1%; and DL-valine, 15.7%.

[4] Supplied by American Lecithin Company, Woodside, L. I., N. Y.

[5] Salt mixture of Clark ('27) plus the following trace minerals per liter of liquid milk: $FeSO_4 \cdot 7H_2O$, 50 mg; $CuSO_4 \cdot H_2O$, 4 mg; $CoCl_2 \cdot 6H_2O$, [illegible].4 mg; $MnSO_4 \cdot H_2O$, 3.4 mg; $ZnSO_4 \cdot 7H_2O$, 3.1 mg; and KI, 0.3 mg.

[6] Vitamins added to each liter of "liquid" diet included: thiamine hydrochloride, 0.65 mg; riboflavin, 0.65 mg; niacin, 2.50 mg; inositol, 26.0 mg; choline, 260.0 mg; PABA, 2.60 mg; folic acid, 0.052 mg; pyridoxine hydrochloride, 0.65 mg; calcium pantothenate, 1.30 mg; mixed tocopherols, 1.2 mg; menadione, 0.28 mg; ascorbic acid, 10.00 mg; vitamin $B_{12}$ (supplied by Merck and Co.), 8 $\mu$g; vitamin A, 2,000 I.U., and vitamin D, 200 I.U., both supplied by a concentrated fish liver oil.

levels of total L-threonine in the various diets are shown in table 2 along with the amounts of DL-threonine fed.

Originally 5 pigs were placed on each of the experimental diets; however, one pig on the diet containing 0.73% L-threonine failed to gain over the experimental period. Autopsy showed this to be due to an umbilical hernia which resulted in a constriction of the prolapsed intestine, causing severe intermittent bloating. Since the inability of this pig to make normal gains was due purely to an anatomical disorder, the data on this animal were discarded.

TABLE 2

*Average data for pigs receiving various levels of threonine*

| | SUPPLEMENTAL LEVEL OF DL-THREONINE (%) | | | | |
|---|---|---|---|---|---|
| | None | 0.366 | 0.746 | 1.127 | 1.515 |
| Total L-threonine in diet (%) | 0.36 | 0.54 | 0.73 | 0.92 | 1.12 |
| Number of pigs | 5 | 5 | 4 | 5 | 5 |
| Initial wt. (kg) | 1.81 | 1.88 | 1.92 | 1.91 | 1.97 |
| Final wt. (kg) | 1.98 | 2.61 | 4.18 | 4.79 | 4.45 |
| Daily gain (gm)[1] | 8.0±1.7 | 34.0±1.7 | 107.0±3.0 | 137.0±3.8 | 118.0±3.6 |
| Daily gain (% of initial wt.) | 0.44 | 1.80 | 5.57 | 7.17 | 5.99 |
| Dry matter intake/kg gain (kg) | 12.33 | 4.12 | 1.90 | 1.54 | 1.79 |

[1] Mean ± standard error of the mean.

The data reported in table 2 clearly show that the basal diet is deficient in threonine, as the pigs did little more than maintain their initial weights. Increasing the level of threonine in the diet produced highly significant [2] differences in average daily gains, showing progressive increases up to the 0.92% level of L-threonine. The marked differences in the size of the pigs fed various levels of threonine are illustrated in figure 1. Raising the L-threonine content of the diet to 1.12% did not increase the growth rate above that obtained on the 0.92% level. The efficiency of feed utiliza-

[2] Analysis of covariance.

tion was correspondingly increased with each increase of threonine up to the 0.92% level, but was not further improved by additional threonine. From these data it appears that the level of L-threonine necessary for maximum growth and feed efficiency of suckling pigs fed a diet containing 25% protein is approximately 0.92% of the air dry matter of the diet. This is equivalent to 3.7% of the dietary protein. A calcu-

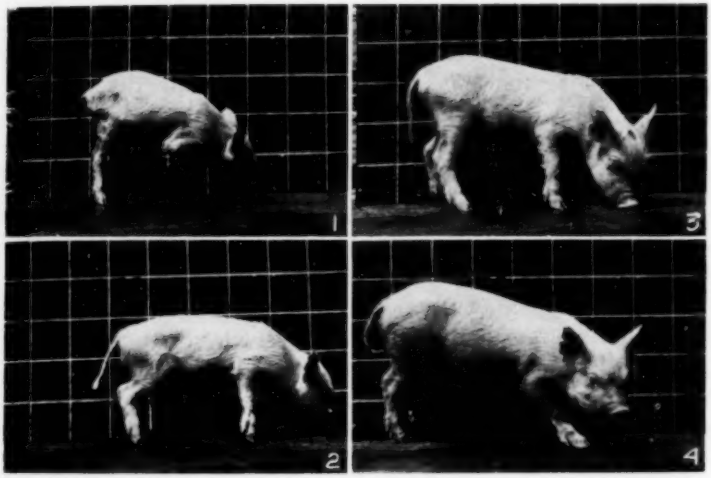


Fig. 1 These pigs illustrate the striking growth response obtained from added threonine. Pig 1 received 0.36% of the air dry matter of the diet as L-threonine, pig 2 received 0.54% L-threonine, pig 3 received 0.73% L-threonine and pig 4 received the 0.92% level of L-threonine.

lated peak was derived by fitting a cubic equation to the data (Snedecor, '46), and using the partial regression coefficients obtained in a prediction equation to calculate the peak value. This mathematical treatment indicates a requirement of 3.8% of the protein as L-threonine, or approximately 0.95% of the air dry matter of the diet. The data from this study are too few to warrant complete reliance on the calculated maximum value, but the method appears useful in in-

creasing the accuracy of interpretation of experiments of this type.

## DISCUSSION

It should be recognized that the L-threonine requirement of approximately 0.9% of the diet applies only to pigs of this age and weight group, and particularly to rations containing approximately 25% protein.

It is of interest to note that the requirement by swine of 0.9% threonine in the diet is somewhat higher than the 0.6% requirement of threonine reported for the chick (Almquist et al., '47) or the 0.5% level required by the rat (Rose et al., '52). However, when these values are expressed as percentages of the dietary protein, the differences between species are eliminated to a great extent, becoming requirements of 3.6% of the dietary protein for the suckling pig as compared to approximately 3.0% for the chick and 3.3% for the weanling rat.

The importance of considering the level of dietary protein in evaluating the amino acid requirement of pigs has been stressed in the earlier work of Brinegar et al. ('50b). This is especially true in view of the variability of the recommended protein allowances for this species at the different stages of growth.

## SUMMARY

Suckling pigs were fed simulated milk diets containing approximately 25% protein on a dry matter basis. Dietary nitrogen was supplied by isolated soybean ("alpha") protein, crystalline amino acids and ammonium citrate (dibasic). Growth and feed efficiency were used as criteria in studying the effects of adding various levels of DL-threonine to this diet over a three-week period.

The data suggest that the L-threonine requirement is approximately 0.9%. This is equivalent to 3.6% of the dietary protein.

ACKNOWLEDGMENTS

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# EFFECT OF CHOLINE SUPPLEMENTS ON FATTY METAMORPHOSIS AND LIVER CELL DAMAGE IN CHOLINE AND PROTEIN DEFICIENCY [1]

DIETER KOCH-WESER,[2] J. DE LA HUERGA AND HANS POPPER

*Hektoen Institute for Medical Research and Departments of Pathology of the Cook County Hospital and Northwestern University Medical School, Chicago, Illinois*

TWO FIGURES

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Ever since the lipotropic activity of choline was discovered (Best et al., '33; Best and Huntsman, '34), its role in prevention and treatment of liver disease, and especially of fatty liver, has been argued (Best et al., '52). Considerable attention has been given to the relation between available choline and general protein deficiency (Best and Lucas, '43; Treadwell, '48) and to the influence of pure fatty metamorphosis upon liver function (Koch-Weser et al., '51). Moreover, it is still questionable whether in liver damage the choline requirement is increased (Best et al., '52), although apparently the choline absorption from the intestine is, if anything, increased in the presence of experimental liver damage (Popper et al., '52). The present study deals with histological and chemical criteria of liver damage in rats fed diets low in protein or in choline. The effect of high therapeutic doses of choline was to be tested in both groups.

[1] This study was supported by a grant from the Dr. Jerome D. Solomon Memorial Research Foundation.

[2] Present address: Department of Medicine, University of Chicago, Chicago, Illinois.

## MATERIAL AND METHODS

A total of 83 Wistar strain female rats weighing 175 to 228 gm were used. Groups 1 and 2, consisting of 33 rats, were kept for 5 weeks on a choline-deficient diet (which is also low in methionine) consisting of 20% peanut meal extracted 5 times with hot ethyl alcohol, 6% casein, 3% corn oil, 2% cod liver oil, 65% dextrose and the following vitamin supplements per kilogram of diet: thiamine hydrochloride, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 40 mg; riboflavin, 20 mg; nicotinic acid, 40 mg; biotin, 0.01 mg; oleum percomorphum, U.S.P., 1 ml. To conform with previous experiments with these diets in this laboratory, vitamin $B_{12}$ and folic acid were not added. The choline content of the extracted peanut meal was determined (Handler, '47) and found to be 0.6 mg/gm. At the end of 5 weeks, 11 of these rats (group 2) received 30 mg of choline chloride by stomach tube in two doses 16 hours apart. Together with the 22 untreated animals on the same diet (group 1), they were killed by bleeding from the abdominal aorta 40 hours after the first doses.

Groups 3 and 4, consisting of 40 rats, were kept for 4 weeks on a low protein, high fat diet with adequate supplementation of choline. The diet consisted of 6% casein, 38% hydrogenated cottonseed oil, 5% cod liver oil, 49% sucrose and the same vitamin supplements as for groups 1 and 2 with the addition of 1.2 gm of choline chloride per kilogram of diet. The average food intake in groups 3 and 4 was 4.8 gm per day per animal, corresponding to an intake of 5.8 mg of choline chloride per day per animal, an amount adequate to prevent fatty liver under normal circumstances (Channon et al., '38). At the end of 4 weeks 13 of these rats (group 4) received 30 mg of choline chloride in the same way as group 2. They were killed, together with the 27 untreated animals on the same diet (group 3), 40 hours after the first doses.

As controls, 10 animals (group 5) on a well-balanced laboratory diet [3] were killed. Histologic preparations of the liver stained with hematoxylin-eosin and Sudan IV were examined and the following biochemical tests were performed:

### *A. In liver tissue*

*Total lipids.* Of liver tissue, ground with anhydrous sodium sulfate, an aliquot was extracted with chloroform and re-extracted with petroleum benzin U.S.P. The lipid residue was weighed after removal of the petroleum benzin U.S.P. The results were recorded in grams per 100 gm of wet liver tissue.

*Alkaline phosphatase.* Liver tissue was kept frozen and subsequently homogenized. Alkaline phosphatase was determined (Bodansky, '33) using sodium β-glycerophosphate as substrate. The activity of this enzyme was recorded in micromoles (uM) per hour per 100 gm wet liver tissue.

*Esterase.* In the same homogenate the activity was determined with phenylbenzoate as substrate (Gomori, '49) and its activity recorded in millimoles (mM) per hour per 100 gm of wet liver tissue.

### *B. In serum*

*Bromsulphthalein retention.* Thirty minutes before the animal was killed by bleeding from the abdominal aorta, 5 mg of bromsulphthalein sodium per 100 gm of body weight were given intraperitoneally. The concentration of the dye in the serum was expressed in milligrams per 100 ml of serum.

*Alkaline phosphatase.* This was determined by the same method as alkaline phosphatase in the liver homogenate (Bodansky, '33) and expressed in micromoles per hour per 100 ml serum.

*Esterase.* This was determined by the same method as esterase in liver homogenate (Gomori, '49) and expressed as millimoles per hour per 100 ml serum.

[3] Purina dog chow checkers.

*Bilirubin.* This was determined according to the method of Rappaport ('49) and recorded as milligrams per 100 ml serum.

## RESULTS

After 4 weeks on a choline-deficient diet, fed ad libitum, the animals had lost an average of 15% of their original weight. The livers were large and uniformly yellow. Microscopically there was diffuse fatty metamorphosis (infiltration), the fat being accumulated in relatively large globules in the liver cells of all zones. The nuclei were as a rule pushed to the side, but fatty cysts (Hartroft, '50) were not seen. Cytoplasm and nuclei of the non-fatty cells appeared normal. There was no necrosis (fig. 1 a).

Chemically no significant difference from the controls was found in hepatic and serum esterase and alkaline phosphatase. Bromsulphthalein (BSP) retention, serum bilirubin and the total liver lipids were increased significantly without overlapping with the control values (fig. 2).

After 4 weeks on a low protein diet the rats had lost 28% of their weight. The livers were large, brown-yellowish and their surfaces somewhat mottled. Microscopically, the fat was distributed throughout the lobule in smaller intracellular globules. The cytoplasm of the non-fatty liver cells showed marked variations in staining qualities and numerous focal necroses were present (fig. 1 c).

Fig. 1 Photomicrographs of rat livers. Hematoxylin-eosin stain. × 120.

a. Choline-deficient diet, 5 weeks. The liver cells contain mostly large fat droplets displacing the cytoplasm. No evidence of other liver cell damage is seen.

b. Choline-deficient diet, 5 weeks, then 15 mg of choline orally 48 and 32 hours before death. Some of the liver cells contain small fat droplets, but reveal otherwise no abnormalities.

c. High fat, low protein diet, 4 weeks. Most of the liver cells contain large fat droplets displacing nucleus and cytoplasm. The fat-free liver cells exhibit irregular clumping of the cytoplasm. Some of the liver cells are missing and scavenger cells accumulate in their place.

d. High fat, low protein diet, 4 weeks, then 15 mg of choline orally 48 and 32 hours before death. The liver cells almost free of fat; however, the cell plates are irregularly arranged, and the cytoplasm and nuclei of liver cells vary in staining qualities, the former being clumped in several places. Small focal necroses are noted.

Hepatic and serum alkaline phosphatase activities were uniformly increased and that of esterase decreased. Bromsulphthalein retention, serum bilirubin and total liver lipids showed similar changes to those observed in the choline-

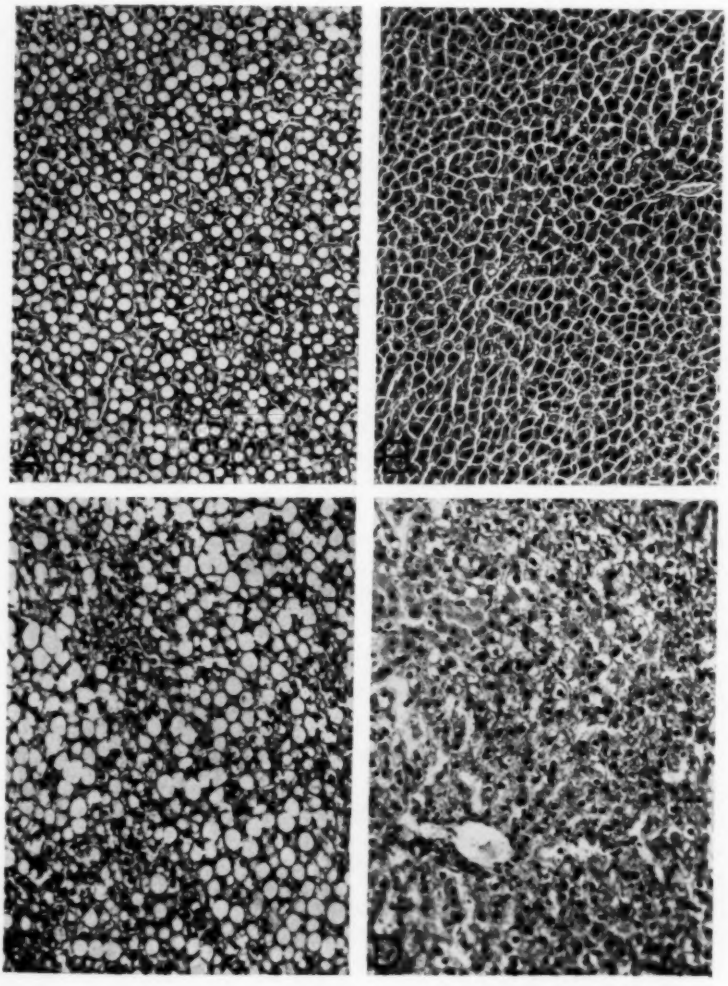

Figure 1

deficient group (fig. 2). Forty hours after the first doses of choline, the hepatic fatty changes of the choline-deficient animals had almost completely subsided (fig. 1 b) and BSP retention, serum bilirubin and total liver lipid concentration

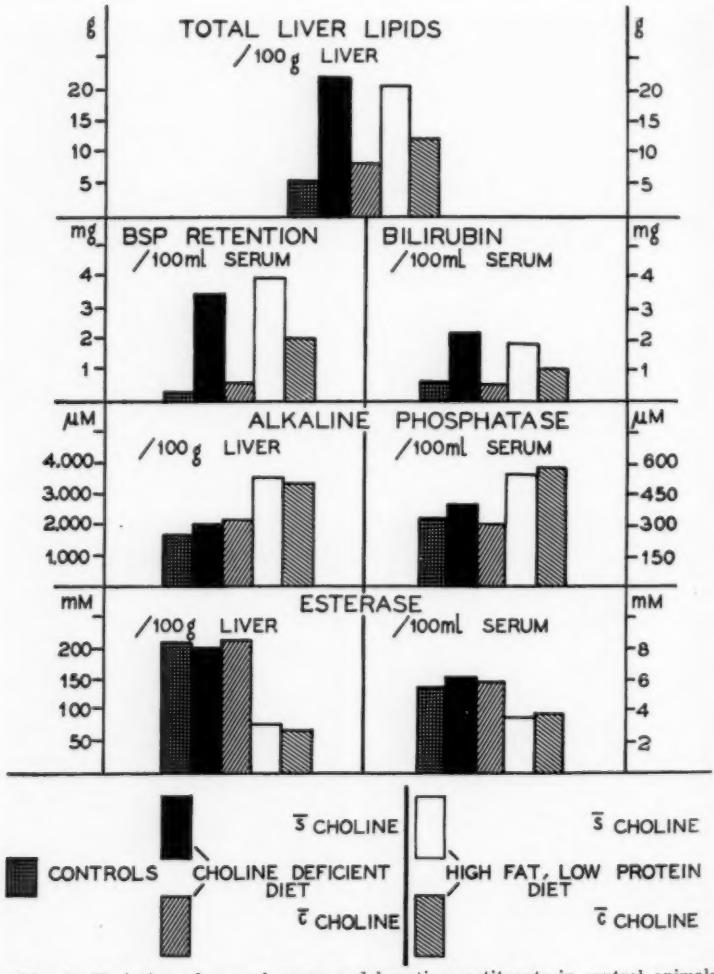


Fig. 2 Variation of several serum and hepatic constituents in control animals as well as in rats on choline-deficient and high fat, low protein diets, with and without preterminal choline administration.

became almost normal. The enzyme values, normal before choline administration, remained unchanged (fig. 2).

When the same amount of choline was given to the animals on a low protein diet, large amounts of fat were removed, as proven histologically (fig. 1 d) and chemically (fig. 2). The BSP retention and serum bilirubin were still slightly increased, while alkaline phosphatase in liver and serum remained high and esterase low.

In general, where the means, illustrated by columns in figure 2, revealed obvious variations, the differences in the findings were uniform and no overlapping with the control values was observed.

## DISCUSSION

Previous investigations (Koch-Weser et al., '51) have indicated that increase of alkaline phosphatase and decrease of esterase in liver tissue and serum reflect liver cell damage, while increase in serum bilirubin and BSP retention may be due to hepatic fatty metamorphosis alone. These criteria were used in conjunction with the histologic findings to evaluate differences between the results of protein deficiency and those of choline deficiency.

In the early stage, choline deficiency brings about a pure fatty metamorphosis which seems to lead to the later developing cirrhosis (Hartroft and Ridout, '51). In this early stage the marked fatty metamorphosis is not accompanied either by morphological signs of damage in the non-fatty liver cells or by disturbance of the liver function as expressed in enzyme determination. As in another acute fatty metamorphosis, after intraperitoneal administration of ethionine (Farber et al., '50, '51) the only altered findings are increased BSP retention and serum bilirubin, both probably due to impairment of the hepatic blood and bile flow rather than to disturbed excretory function (Koch-Weser et al., '51). In contrast, a protein deficiency with adequate choline supply causes not only fatty metamorphosis, but marked liver damage. This lesion differs by the absence of massive necrosis

from the well-known condition brought about by deficiency of the sulfur amino acids and vitamin E. By treating this condition with high doses of choline, it was possible to dissociate the fatty metamorphosis from the liver cell damage.

Protein deficiency is known to bring about, besides liver cell damage, marked fatty metamorphosis, even with an adequate choline supply. A large part of this fat can be removed by high doses of choline (Best and Lucas, '43; Treadwell, '48). These observations may be important in relation to the question of whether or not choline supplementation is indicated in liver disease (Volwiler et al., '48; Franklin et al., '48; Post et al., '52; Cayer and Cornatzer, '52), provided that the protein intake is insufficient. It seems that the choline requirement is higher in liver damage produced by protein deficiency as far as lipotropic action is concerned. It should, however, be stressed that the removal of fat does not relieve the damage produced by protein deficiency.

Whether liver damage as such or protein deficiency is the determining factor remains to be decided. Anyway, it appears that under the conditions of these experiments large supplements of choline are effective in removing fat (thus possibly preventing development of fatty cirrhosis), but not in correcting the present liver damage.

## SUMMARY

Histologic and biochemical characteristics of liver cell damage and fatty metamorphosis were studied in rats on choline-deficient diets (with adequate choline supply), in both instances with and without supplementation with large doses of choline. Elevation of serum and hepatic phosphatase and reduction of serum esterase were considered chemical indications of liver cell damage, whereas BSP retention and increased bilirubinemia run parallel with fatty metamorphosis.

Rats in early choline deficiency reveal no chemical or histologic evidence of liver cell damage except fatty metamorphosis, the latter being corrected by choline supplementation. Fol-

lowing a high fat, low protein diet chemical and histologic evidence of both fatty metamorphosis and liver cell damage is found. Choline supplements remove large parts of the excessive lipids and their manifestations but do not influence the evidence of liver cell damage.

It appears that large choline supplements remove fat in protein deficiency with adequate intake of choline, thus probably preventing development of fatty cirrhosis, but do not influence the liver cell damage present.

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# GROWTH STIMULATION AND GROWTH INHIBITION OF CHICKS FED FORAGE AND FORAGE JUICE CONCENTRATE

R. G. HANSEN,[1] H. M. SCOTT,[2] B. L. LARSON,[3] T. S. NELSON[4] AND P. KRICHEVSKY[5]

*Illinois Agricultural Experiment Station, Urbana*

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Spitzer and Phillips ('46) observed that supplementing a corn-soybean diet containing 5% alfalfa meal with an additional 10% alfalfa meal increased the percentage of rats weaned. Bowland et al. ('48), using a purified diet, have presented evidence that dehydrated alfalfa meal contains an unidentified growth factor or factors for the rat; further, 15% alfalfa meal improved the ability of females to wean their young. However, this level did not contain all of the factors or a sufficient amount of them to maintain normal lactation.

Alfalfa meal has been shown to depress chick growth under certain conditions (Roberts et al., '39; Draper, '48; Cooney et al., '48; Insko and Culton, '49; Lepkovsky et al., '50; Peterson, '50a, b; Heywang, '50; German and Couch, '50; Kodras et al., '51a, b). A growth-limiting principle which simulated the effects of purified saponins has been extracted from alfalfa meal with hot water, leaving a residue which was quite innocuous to the chick (Peterson, '50a). The ingestion of cholesterol or certain plant sterols with a vegetable oil largely neutralized the growth depression resulting from either the extract or the purified saponin

[1] Laboratory of Biochemistry, Department of Dairy Science.
[2] Department of Animal Science.
[3] Laboratory of Biochemistry, Department of Dairy Science.
[4] Department of Animal Science.
[5] Laboratory of Biochemistry, Department of Dairy Science.

(Peterson, '50a, b). These results have been interpreted as indicating that the saponin content limits the amount of alfalfa that can be fed to chicks. Inasmuch as the residue remaining after the hot water extraction did not inhibit growth, fiber per se does not appear to be the growth limiting principle (Lepkovsky et al., '50).

The use of alfalfa in chick starting rations has been studied at the Illinois Agricultural Experiment Station (Roberts et al., '39), and the more recent results have been summarized by Card ('51). Subsequently, attention has been drawn to a sample of field-cured hay containing some alfalfa that had reputedly caused cows and horses to salivate profusely. Under practical conditions the symptoms of salivation presumably resulted from the ingestion of quantities of this forage. The hypothesis that an excess of saponins might cause these larger farm animals to salivate profusely deserves consideration in view of their known effect on surface tension. The sensitivity of the chick to saponins in the diet suggested that this species might be used to determine the approximate amount of saponins in a variety of forage samples. Experiments were therefore designed in which sun-cured and dehydrated meals were added to the diet of chicks either as part of a semi-purified diet or as part of a natural diet. The results obtained in the first phase of the investigation prompted further trials with additional meals. For obvious reasons it seemed desirable also to compare the forages with a forage juice concentrate.

## EXPERIMENTAL AND RESULTS

The sample of forage which had allegedly produced salivation in the larger farm animals had been cured in the sun and was composed of timothy, lespedeza, clover and only a small part of alfalfa. Throughout this report it is designated as the sun-cured meal (sample X). An immediate problem was that of a meal to use for comparison. A sample of good quality dehydrated alfalfa meal (sample Y), obtained

on the open market, was chosen for this purpose because of the availability of quantities of a standard product. The sun-cured meal was ground first in the hammer mill, then through a Wiley mill using a one-twenty-fifth screen. Two types of basal diets were used for this study, the details

TABLE 1

*Composition of diets*

| SEMI-PURIFIED (BASAL 1) | AMOUNT | NATURAL (BASAL 2) | | PER 100 LB. |
|---|---|---|---|---|
| | % | | | % |
| Cerelose | 62.36 | Ground yellow corn | | 52.55 |
| Casein, vitamin-free | 18.00 | Soybean meal, 50% protein | | 38.50 |
| Gelatin | 10.00 | Dicalcium phosphate | | 3.00 |
| Corn oil | 4.00 | Limestone | | 1.00 |
| Salts [1] | 5.34 | Corn oil | | 4.00 |
| DL-Methionine | 0.30 | Iodized salt | | 0.50 |
| | | A and D oil (3000 A, 600 D) | | 0.30 |
| Total [2] | 100.00 | Manganese sulfate-feed grade | | 0.05 |
| | | DL-Methionine | | 0.10 |
| | | $B_{12}$ | 0.4 mg | + |
| | | Riboflavin | 0.2 gm | + |
| | | Ca-pantothenate | 0.3 gm | + |
| | | Choline Cl | 30.0 gm | + |
| | | Niacin | 1.0 gm | + |
| | | Aureomycin | 2.2 gm | + |
| | | | Total | 100.00 |

[1] Glista ('51).

[2] Plus the following vitamins (milligrams per kilogram diet): Thiamine HCl, 4.0; riboflavin, 16.0; calcium pantothenate, 20.0; choline Cl, 2,000.0; niacin, 100.0; pyridoxine HCl, 6.0; biotin, 0.6; folic acid, 4.0; inositol, 1,000.0; para-aminobenzoic acid, 2.0; menadione, 1.0; $B_{12}$, 0.02; vitamins A, D and E given weekly by dropper.

of which are given in table 1. In the semipurified diet the meals were substituted at the expense of cerelose, while in the natural diet this substitution was made for yellow corn.

Day-old chicks originating from a cross of Columbian males to New Hampshire females were used in all experiments. The breeding stock had received a diet containing liberal amounts of animal protein and dehydrated alfalfa. The

chicks were selected at random from the hatching trays to make up the various lots. The lots were randomized in the compartments of an electrically heated battery equipped with raised wire floors. Feed and water were offered ad libitum. The chicks were weighed individually at weekly intervals. Feed consumption data were recorded for each 7-day period.

The results obtained with the original dehydrated meal (sample Y) and the sun-cured meal (sample X) are presented in table 2. It is apparent that as much as 20% of either forage when added to a purified diet failed to depress chick growth. Forty per cent of either meal did depress growth, but only the sun-cured meal was significantly different from the basal ration in this respect. Chicks fed the diet containing 40% dehydrated meal grew as well as or better than the basal chicks for the first three weeks. Only after the third week did growth decline sharply. With the 40% level of sun-cured meal the growth depression became evident at a somewhat earlier age. Of equal interest is the fact that chick growth was accelerated by both the dehydrated and the sun-cured products. With the former meal an improvement in growth was observed with each increment of dehydrated meal up to the 20% level, but it did not reach statistical significance until 10% was added to the basal. It is also clear that the sun-cured meal was definitely superior to the dehydrated meal in promoting chick growth. In contrast with the dehydrated meal, it will be observed that each increment of sun-cured meal above 5% resulted in some decline in growth. It would appear from the data presented in table 2 that: (1) as much as 20% of either dehydrated or sun-cured meal can replace the cerelose in a purified diet without inhibiting chick growth; (2) that both types of meal contain a factor, probably unidentified, that improves chick growth; and (3) that the sun-cured meal was a better source of the factor than the dehydrated meal.

No previous published work has come to our attention in which a purified chick diet was used in studying the alfalfa meal problem. Without exception the work previously cited

has been carried out with the practical type of diets. Since the forage material failed to depress chick growth when supplementing our purified basal diet, except at the 40% level, the original samples of meals X and Y were incorporated in a diet (table 1) composed of natural feed ingredients. Two experiments were run in which the diets were alike except that 4% corn oil was present in one and omitted in the other. This was done because it has been shown that certain vegetable oils will alleviate to some extent the growth

TABLE 2

*The effect on chick growth of adding graded amounts of dehydrated alfalfa meal and sun-cured hay meal to a purified diet* [1]

| WEEKS | BASAL | SUPPLEMENT TO BASAL 1 (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Dehydrated meal (sample Y) | | | | Sun-cured meal (sample X) | | | |
| | | 5 | 10 | 20 | 40 | 5 | 10 | 20 | 40 |
| 1 (gm) | 69 | 72 | 80 | 82 | 76 | 91 | 83 | 89 | 70 |
| 2 (gm) | 130 | 140 | 151 | 167 | 144 | 171 | 156 | 150 | 126 |
| 3 (gm) | 213 | 229 | 246 | 243 | 213 | 285 | 258 | 258 | 199 |
| 4 (gm) | 302 | 317 | 332 | 337 | 279 | 391 | 374 | 346 | 258 |
| "t" value [2] | | 1.17 | 3.02** | 2.43* | 1.89 | 8.14** | 6.99** | 3.81** | 4.13** |
| Efficiency [3] | 0.51 | 0.56 | 0.52 | 0.54 | 0.43 | 0.58 | 0.58 | 0.52 | 0.38 |

[1] Fifteen chicks per lot, mixed sexes.

[2] In relation to 28-day weight of basal chicks.

* Significant at the 5% level of confidence.

** Significant at the 1% level of confidence.

[3] Grams gain per gram feed consumed.

depression resulting from high concentrations of alfalfa meal in the chick diet. With regard to growth depression, the results of this experiment with the practical ration are quite similar regardless of the presence of corn oil (table 3). In the presence of corn oil the 20% level of either meal significantly depressed growth below that obtained with the basal ration. It is also readily apparent that neither sample of meal fed to chicks at the 5% or 10% level resulted in chick weights that differed significantly from the weight of the basal chicks. When corn oil was omitted, the results

were essentially the same in that both the dehydrated and sun-cured meals depressed growth at the 20% level. There is some suggestion that the 5 and 10% levels of both meals improved growth but only the 5% level of dehydrated meal differed significantly from the basal ration in this respect. The absolute growth of the lots receiving either meal at the 20% level, irrespective of the corn oil, was surprisingly good in comparison with the values reported by others who

TABLE 3

*Effect of different levels of dehydrated alfalfa and sun-cured meal on chick growth when added to a natural diet with and without corn oil* [1]

| CORN OIL | | BASAL | SUPPLEMENT TO BASAL 2 (%) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Dehydrated meal (Y) | | | Sun-cured meal (X) | | |
| | | | 5 | 10 | 20 | 5 | 10 | 20 |
| % | | | | | | | | |
| None | Weight [2] | 355 | 387 | 368 | 340 | 373 | 368 | 311 |
| | "t" value [3] | | 2.90** | 1.17 | 1.17 | 1.78 | 1.09 | 4.46** |
| | Efficiency [4] | 0.52 | 0.52 | 0.49 | 0.44 | 0.50 | 0.45 | 0.39 |
| 4.0 | Weight [2] | 386 | 369 | 378 | 355 | 372 | 381 | 341 |
| | "t" value [3] | | 1.19 | 0.56 | 2.32* | 1.24 | 0.43 | 4.15** |
| | Efficiency [4] | 0.58 | 0.55 | 0.54 | 0.51 | 0.56 | 0.52 | 0.43 |

[1] Twenty chicks per lot, mixed sexes.
[2] Grams at 28 days.
[3] In relation to weight of basal chicks.
* Significant at the 5% level of confidence.
** Significant at the 1% level of confidence.
[4] Grams gain per gram feed consumed.

have fed this level to chicks for 28 days.

As previously explained, the sample of sun-cured meal used in this experiment contained a mixture of forages. While improbable, it is nevertheless possible that any one or a combination of several of these forages other than alfalfa would result in a meal having greater growth-promoting power than the dehydrated alfalfa meal. To rule out the possibility that the results obtained in the first experiment were peculiar to these two samples, additional samples of alfalfa meal (samples A, B, C, and D) were obtained from

commercial producers. Samples A and C were dehydrated under commercial conditions. Samples B and D were sun-cured. In addition, a forage juice concentrate (Kohler and Graham, '51) was included for comparison.[6] All samples were added to the purified basal ration at the 5% level. Each of the supplements significantly improved growth over that obtained with the chicks receiving the basal ration (table 4). Both samples of dehydrated alfalfa meal, the

TABLE 4

*Stimulation of chick growth by forage juice concentrate and different samples of dehydrated and sun-cured alfalfa meal when incorporated in a purified diet* [1]

| | BASAL | SUPPLEMENT TO BASAL 1 (5.0%) | | | | |
|---|---|---|---|---|---|---|
| | | Forage juice | Dehydrated meal | | Sun-cured meal | |
| | | | A | C | B | D |
| Weight [2] | 292 | 347 | 333 | 332 | 345 | 384 |
| "t" value [3] | | 3.14** | 2.63* | 2.95** | 3.72** | 6.25** |
| Efficiency [4] | 0.48 | 0.50 | 0.54 | 0.54 | 0.54 | 0.58 |

[1] Twenty chicks per lot, mixed sexes.
[2] Grams at 28 days.
[3] In relation to weight of basal chicks.
* Significant at the 5% level of confidence.
** Significant at the 1% level of confidence.
[4] Grams gain per gram feed consumed.

B sample of sun-cured meal and the juice concentrate resulted in comparable 28-day chick weights. Sun-cured sample D was, however, significantly superior to the other supplements in promoting chick growth. The existence of a growth factor required by the chick in dehydrated and sun-cured alfalfa meal is thus confirmed.

## DISCUSSION

When graded levels of alfalfa meal were added to a purified diet, complete in all known nutrients required for chick growth, it became apparent that alfalfa meal contains one

[6] Supplied by Cerophyl Laboratories, Inc.

or more unidentified growth factors, as well as a factor which inhibits chick growth. The growth-inhibiting factor, presumably a saponin, tends to mask the growth-stimulating factor when high levels of alfalfa meal are incorporated in the purified diet.

It is difficult to demonstrate that alfalfa meal will stimulate chick growth when fed with a natural diet. This would suggest that the factor present in alfalfa meal is also found in other feed ingredients commonly used to formulate practical chick rations. Growth inhibition, on the contrary, can be more readily demonstrated on a natural diet than on a purified diet. A possible explanation for this is that plant materials other than alfalfa meal may contribute to the sum total of inhibitory substances, since it is well-known that saponins are widely distributed in plant tissues, as, for example, in soybean oil meal.

Kohler and Graham ('51) have presented evidence regarding a "grass juice factor" which improves chick growth. They have shown that the factor, in green material such as cereal grass and alfalfa, is destroyed by high temperature. They were unable to demonstrate its activity in dehydrated vegetation. The data presented here would seem to indicate that the high temperature (1,500 to 1,800°F.) used by commercial dehydrators does not completely destroy this activity, although it may affect it somewhat. A direct comparison of dehydrated cereal grass (oat) and forage juice concentrate is presented in table 5. The response to dehydrated cereal grass was comparable to that which might be expected to dehydrated alfalfa meal. Forage juice was superior to the cereal grass but not significantly so, and a combination of forage juice and cereal grass gave no better growth than the juice concentrate alone. The positive control group fed the sun-cured hay meal (sample X) responded as well as the group receiving the forage juice concentrate. Whether the factors producing the present response are similar to

the "grass juice factor" must await further experimentation.

Evidence has been presented showing that sun-cured meals may elicit a growth response that is definitely superior to that noted for the dehydrated meals with which they were compared. In the experiments reported here this was true in two of three instances (tables 2 and 4). One sample of sun-cured alfalfa meal (B) failed to give a growth response that differed significantly from that obtained with the dehydrated meals (table 4). It should be pointed out that all of the sun-cured meals were from the 1951 spring cutting,

TABLE 5

*Growth response of chicks fed dehydrated cereal grass and forage juice concentrate* [1]

| SUPPLEMENT TO BASAL | WEIGHT [2] | EFFICIENCY [3] |
|---|---|---|
| None | 307 | 0.54 |
| 5% dehydrated cereal (oat) grass | 333 | 0.58 |
| 5% forage juice concentrate | 348 | 0.53 |
| 5% cereal grass + 5% forage juice concentrate | 343 | 0.56 |
| 5% sun-cured meal (sample X) | 350 | 0.57 |

[1] Twenty-five chicks per lot, mixed sexes.

[2] Grams at 28 days. All treatments were significantly different from the basal at the 5% level of confidence or better.

[3] Grams gain per gram feed consumed.

while the dehydrated meals, except for sample Y, were known to have been harvested and processed in the spring of 1952. Data concerning the conditions that prevailed during the drying and storage of the sun-cured meals are not available. These sun-cured meals were held in storage for approximately one year before they were made available for this work. It is possible that sun-cured meal subjected to excessive leaching from rainfall during the curing process may loose considerable activity. That this holds true for pantothenic acid and niacin has been demonstrated by Blaylock et al. ('50).

It could be argued that the growth response obtained when the purified diet was supplemented with alfalfa resulted

from the cellulose that the meals contributed to the diet. Davis and Briggs ('47, '48) have submitted evidence that cellulose improves chick growth when added to a purified diet. However, unpublished data from this laboratory do not substantiate the findings of Davis and Briggs. Neither are their results in harmony with the evidence presented by Scott et al. ('47). That the response noted here is quite independent of the cellulose contributed by the meals is demonstrated by the ability of plant extracts, virtually free of cellulose, to elicit the same growth response as the dehydrated alfalfa meal. Furthermore, it is unlikely that these results can be attributed to additional protein supplied by the forage meal and forage juice concentrate, inasmuch as the basal diet supplied adequate amounts of the essential amino acids required by the chick (Glista, '51).

The ability of chicks to tolerate a higher level of dehydrated meal than of sun-cured meal before growth starts to decline warrants consideration. If the growth inhibition is due solely to saponins, then it would be reasonable to assume that the sample of sun-cured meal used in the first experiment (table 2) contained more of this compound than did the dehydrated meal. The bulkiness of the diet per se may have prevented the chicks from consuming a sufficient amount of the diet to meet their requirement for the various nutrients and particularly for energy. Jensen ('49) has shown that the growth inhibition resulting from feeding chicks high concentrations of alfalfa meal in a mash ration could be alleviated appreciably by pelleting the same diet to increase its density. That this is not peculiar to alfalfa diets was demonstrated by Scott et al. ('47), since they improved chick growth by increasing the density of a high oat diet by merely reducing the particle size of the ground oats in a Wiley mill.

## SUMMARY AND CONCLUSIONS

Chick growth was stimulated when either dehydrated or sun-cured alfalfa was added to a purified diet containing all

known factors. Dehydrated meal at a level of 10 to 20% gave a response comparable to that elicited by 5% of a forage juice concentrate. The factor appears to be more concentrated in sun-cured meal than in dehydrated meal, since the former gave its maximum response at the 5% level. This would suggest that the high temperature required to process dehydrated meal was responsible for the decrease in activity. The existence of the growth factor in alfalfa meal is difficult to demonstrate in chicks when the meal is added to a practical diet.

When added to a purified diet, alfalfa meal does not depress chick growth until the level of intake exceeds 20%. Even at a 40% level, alfalfa meal does not materially depress growth below that of chicks receiving no alfalfa. Growth inhibition can be more readily demonstrated with the practical diet than with the purified diet, which suggests that other natural feed ingredients may also contain substantial amounts of the growth inhibitor.

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# OXIDATIVE ENZYMES OF THE LIVER IN PROTEIN DEPLETION [1]

W. W. WAINIO, BERTRAM EICHEL,[2] HERBERT J. EICHEL,[3] P. PERSON,[4] FRANCES L. ESTES AND J. B. ALLISON

*Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey*

EIGHT FIGURES

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The concept of a dynamic state of proteins, of an "ebb and flow" (Whipple, Robscheit-Robbins and Miller, '46; Allison, '50), may be used to explain the loss or gain of nitrogen at different rates in the several compartments of the body. For example, total circulating plasma albumin and gamma globulins decrease rapidly in animals fed a low nitrogen diet, while the alpha globulins do not (Chow, Allison, Cole and Seeley, '45). Blood proteins may take priority over tissue proteins in the depleted animal (Whipple, Miller and Robscheit-Robbins, '47). Of the soft tissues of the body, the cytoplasmic proteins of the intestinal tract and liver are most rapidly reduced (Addis, Poo and Lew, '36; Elman and Heifetz, '41; Kosterlitz and Campbell, '45). A series of

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[2] Present address: 3510th Medical Group, ATRC, Randolph Air Force Base, Texas.

[3] Present address: William Goldman Isotope Laboratory, Division of Biological Chemistry, Hahnemann Medical College and Hospital, Philadelphia, Pa.

[4] Public Health Service Postdoctoral Research Fellow of the National Institute of Dental Research, 1950–52. Present address: Dental Research Unit, Army Medical Graduate School, Walter Reed Medical Center, Washington, D. C.

studies designed to characterize this imbalance further have been organized in our laboratories. These studies have extended to the effects of protein depletion on enzymes and enzyme systems, and the present study is an extension of experiments begun in 1945 by Millman and Allison. Millman ('51) reported on adenosine triphosphatase activity in skeletal muscle, and arginase, succinoxidase and cytochrome oxidase activities in liver. Milch ('50) studied pseudocholinesterase in the plasma of normal and protein-depleted rats.

At least 30 enzymes or enzyme systems have been assayed by various investigators in the tissues of animals whose diets have been partly or wholly free of protein. Only those findings will be discussed that have a bearing on the experiments to be presented in this paper.

The succinoxidase activity of rat and mouse livers is reduced in protein depletion (Elson, '47). This has been verified for the rat by Potter and Klug ('47); Benditt, Steffee, Hill and Johnston ('49); Millman ('51); and Bargoni ('51).

Cytochrome oxidase has been reported to decrease progressively with time in the livers of rats on a protein-deficient diet, and in fact somewhat more rapidly than the liver protein (Benditt, Steffee, Hill and Johnston, '49). Lang ('47) and Millman ('51) found the activity of this enzyme to be unchanged.

Liver D-amino acid oxidase decreases when the rat is fed either a low-protein or a protein-free diet (Axelrod, Swingle and Elvehjem, '42; Kaplanskii, Berezovskaya and Schmerling, '45; Lang, '47; Seifter, Harkness, Rubin and Muntwyler, '48; Benditt et al., '49; Khesin, '50; Vanderlinde and Westerfeld, '50).

A number of investigators have reported on the extreme lability of liver xanthine oxidase in partial and complete protein deficiency (MacQuarrie and Venosa, '45; Lang, '47; Westerfeld and Richert, '49, '50; Miller, '50; Litwak, Williams, Feigelson and Elvehjem, '50).

Uricase activity is lowered by 35% in the livers of rats maintained on a 6% casein diet when compared to livers of

animals maintained on an 18% casein diet containing 0.25% DL-methionine (Williams, Feigelson and Elvehjem, '50).

The present investigation was designed in order to study the effects of feeding a protein-free diet on the activities of 8 oxidative enzyme systems of rat liver. Since rats on a protein-free diet reduce their food intake, both pair-fed and ad libitum-fed controls were employed so as to permit a comparison of the effects of protein depletion and partial food restriction.

## EXPERIMENTAL

### *Animals and diets*

The male albino rats used in these two experiments were obtained from the Wistar Institute. They weighed approximately 250 gm when received. They were kept in isolation for at least one week, during which time they were fed a commercial ration.[5] On the third day the animals were injected once with 60,000 units of penicillin-G in oil. They were then placed in individual metabolism cages and all were fed the complete diet containing 18% casein for 4 days. At the start of the experiment, three groups of 5 rats each were equalized with respect to weight. Five rats were given the protein-free diet (hereafter referred to as the depleted animals) and 5 others were given the complete diet (hereafter referred to as the pair-fed animals). Each rat of the second group was pair fed against a rat of approximately the same weight in the first group. The remaining 5 animals were given the complete diet ad libitum (hereafter referred to as the ad libitum animals). Water was available at all times.

The complete diet contained 18% casein, 13.4% sucrose, 20.2% glucose, 18.2% dextrin, 24.2% lard, 1.7% salt mix (Wesson, '32), 1.0% cod liver oil, 3.3% agar, and the known vitamins. The agar was dissolved in an amount of water equal to one and four-tenths times the weight of the dry constituents. The protein-free diet contained 38.2% glucose.

[5] Purina fox chow.

A daily record of food consumption and a weekly record of body weight were kept during the 7 weeks of each experiment, except that the weights were not recorded at the end of the first week in experiment 1 and at the end of the 5th and 6th weeks in experiment 2. One rat (PD 56, ad libitum) died of unknown causes during the 6th week of the second experiment.

### *Handling of the tissues*

At the end of the 7 weeks of the experiment, three rats (one out of each group) were sacrificed by decapitation on each of 5 successive days. The livers were weighed, a piece was removed for histological examination, and the remainder was divided for the enzyme determinations and the total nitrogen, fat and water analyses. Because of the large number of assays to be performed on each liver, it was inconvenient to prepare a separate homogenate for each enzyme assay. However, a special residue preparation was made from a 2-gm piece of liver for the determination of pyruvate oxidation. The large piece of cold liver (approximately 8 gm) was homogenized for one minute with three volumes of cold distilled water in a micro Waring Blendor which was fitted with an ice-jacket, and then further homogenized in a Ten Broeck glass tissue homogenizer. This 1:4 homogenate of liver was then diluted in various ways, depending on the conditions of the enzyme assay.

### *Analytical methods*

Water was determined by drying homogeneous duplicate samples of the 1:4 homogenate to a constant weight at 95°C.

Total nitrogen was determined by the micro Kjeldahl method, employing selenium oxychloride as a catalyst and boric acid to trap the ammonia. The factor 6.25 was used to convert nitrogen to protein wherever necessary.

The method of Hsaio ('48) was used to determine total liver fat. According to this method, a weighed sample of oven-dried tissue is extracted continuously for one-half hour with 95% ethanol and then for one-half hour with anhydrous ethyl ether.

### *Enzyme assays*

A summary of the methods used for the enzymic assays is presented in table 1. It is to be noted that the determination of pyruvate oxidation is made on an insoluble residue preparation which is only a fraction of the cell. Therefore, it is not possible to determine the oxygen uptake for the whole liver or the intact unit of the liver when pyruvate is the substrate. The only value obtained is the oxygen uptake of the residue prepared from a unit of fresh liver.

## RESULTS

### *Food intake and growth*

In experiment 1 the average intake of the ad libitum animals was 1,550 gm at the end of 7 weeks, and that of the depleted and pair-fed animals was 1,150 gm. The pair-fed control animals when compared to the ad libitum controls were restricted by about 26% by the depleted animals. The total intakes were 1,700 gm and 1,350 gm, respectively, in experiment 2 and the restriction amounted to about 21%.

The depleted rats lost weight steadily in both experiments until at the end of 7 weeks they had lost about 27 and 28%, respectively, of their initial body weights. The ad libitum animals gained 105 gm in experiment 1 and 136 gm in experiment 2. The pair-fed animals gained 58 gm in experiment 1, having a final body weight only 12% less than that of the ad libitum animals, and 110 gm in experiment 2, to attain a weight only 7% less than that of the ad libitum animals.

### *Liver composition*

The analyses for water, total protein, and total fat are presented in table 2. The value for the residue was obtained

TABLE 1

*Enzyme assay methods*

| ENZYME SYSTEM | METHOD | DILUTION OF THE 1:4 HOMOGENATE | MEASUREMENT | pH | TEMPERATURE |
|---|---|---|---|---|---|
| | | | | | °C. |
| Cytochrome oxidase | Eichel, Wainio, Person and Cooperstein ('50) | 1:5 with water | Oxygen uptake | 7.1 | 25 |
| Succinoxidase | Schneider and Potter ('43)[1] | 1:5 with water | Oxygen uptake | 7.3 | 25 |
| Succinic dehydrogenase | Cooperstein, Lazarow and Kurfess ('50)[2] | 1:5 with water | Reduction of ferricytochrome *c* | 7.3 | 25 |
| D-Amino acid oxidase | Axelrod, Sober and Elvehjem ('40)[3] | 2:3 with water | Oxygen uptake | 8.2 | 38 |
| Xanthine oxidase | Axelrod and Elvehjem ('41) | 2:3 with water | Oxygen uptake | 7.4 | 38 |
| DPN-cytochrome *c* reductase | Potter and Albaum ('43)[4] | 1:14 with 0.1 M phosphate pH 7.4; then 1:5 with water | Reduction of ferricytochrome *c* | 7.25 | 25 |
| Uricase | Holmberg ('39)[5] | none | Oxygen uptake | 9.0 | 37.5 |
| Pyruvate oxidation | Green, Loomis and Auerbach ('48) | | Oxygen uptake | 7.3 | 25 |

[1] Modified as follows: 1.4 ml 0.1 M $Na_2HPO_4 \cdot KH_2PO_4$, pH 7.4.

[2] Modified as follows: 0.1 ml homogenate, 0.1 ml 0.5 M Na succinate, 0.1 ml $3 \times 10^{-2}$ M NaCN, 1.7 ml 0.18 M $Na_2HPO_4 \cdot KH_2PO_4$, pH 7.3, 1.0 ml of a solution containing 1 mg cytochrome *c*.

[3] Modified as follows: 0.75 ml 0.25 M $Na_2HPO_4 \cdot KH_2PO_4$, pH 8.2, 0.5 ml 1.0 M DL-alanine.

[4] Modified as follows: 0.55 ml 0.1 M $KH_2PO_4$, 0.1 ml homogenate.

[5] Modified as follows: 0.6 ml 0.05 M Li urate, gas phase $O_2$.

[2] Modified as follows: 0.6 ml 0.05 M Li urate, gas phase $O_2$.



TABLE 2

*Liver composition*

| GROUP | EXPERIMENT 1 | | EXPERIMENT 2 | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Wet weight | Protein | Wet weight | Water | Dry weight | Protein | Fat | Residue |
| | gm | gm | gm | gm | gm | gm | gm | gm |
| Depleted | 9.84 (8.05–11.36) | 1.05 (0.90–1.17) | 9.17 (7.94–10.36) | 5.82 [1] (5.10–6.29) | 3.62 [1] (2.84–4.07) | 0.87 (0.85–0.96) | 2.30 [1] (1.90–2.90) | 0.42 [1] (0.15–0.80) |
| Pair-fed | 9.11 (8.50–9.54) | 1.52 (1.44–1.64) | 10.17 (8.48–13.52) | 6.87 (5.58–9.07) | 3.48 (2.64–4.45) | 1.69 (1.41–2.10) | 0.83 (0.63–0.92) | 0.96 (0.48–1.38) |
| Ad libitum | 11.46 (10.24–12.39) | 1.94 (1.77–2.06) | 10.72 [1] (9.55–11.26) | 7.30 [1] (6.53–7.70) | 3.42 [1] (3.02–3.65) | 1.91 [1] (1.84–2.00) | 0.81 [1] (0.55–1.17) | 0.70 [1] (0.57–0.84) |

[1] These values are the averages of 4 animals; all other values are the averages of 5 animals.

by difference. Only the total protein was determined in experiment 1. Here the average weight of the livers of the depleted animals was probably not significantly different from that of the pair-fed animals. However, both had smaller livers generally than did the ad libitum controls. The total protein was lowest and decreased percentage-wise in the livers of the depleted animals; it was a constant percentage of the total liver weight in the other two groups. The same relationships hold for experiment 2 except that the total liver weights of the ad libitum animals were barely greater than the total liver weights for the other two groups. The other analyses show that the dry weight of the livers of the depleted animals was not reduced in spite of the protein that was lost, and this is a reflection of the fact that there had been a substantial and significant increase in total fat.

*Liver enzymes*

The results of the experiment with cytochrome oxidase are presented in figure 1. The results for all of the enzymes are summarized in table 3. Cytochrome oxidase was not determined in experiment 2, since several preliminary studies had revealed the same changes that are reported here. In one preliminary study employing 10 animals the average unit activity per hour per milligram of protein for the depleted animals was 9% higher than for the pair-fed controls. The total activities per hour per liver were the same for the two groups. There were no ad libitum-fed animals for further comparisons. Figure 1 shows a significant increase in the unit activity of cytochrome oxidase per hour per milligram nitrogen (hereafter referred to simply as the unit activity) when the depleted animals are compared to the pair-fed controls, and no difference in the unit activities when the pair-fed animals are compared to the ad libitum-fed controls. The total activities per hour per liver (hereafter referred to as the total activities) when the depleted rats are compared to the pair-fed animals are the same, but are decreased when either the pair-fed or the depleted animals are com-

TABLE 3

*Enzyme changes summarized*

| ENZYME SYSTEM | UNIT ACTIVITY [1] | | | | TOTAL ACTIVITY [2] | | | |
|---|---|---|---|---|---|---|---|---|
| | Depleted vs. pair-fed | | Pair-fed vs. ad libitum | | Depleted vs. pair-fed | | Pair-fed vs. ad libitum | |
| | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| | % | % | % | % | % | % | % | % |
| Cytochrome oxidase | + 33 | | — 16 | | — 7 | | — 34 | |
| Succinoxidase | — 61 | | + 50 [3] | | — 71 | | + 16 | |
| Succinic dehydrogenase | — 58 | — 57 | + 17 | — 8 | — 70 | — 77 | — 9 | — 19 |
| D-Amino acid oxidase | — 37 | — 35 | + 5 | + 37 | — 59 | — 67 | — 18 | + 23 |
| DPN-cytochrome *c* reductase | — 53 | — 59 | — 10 | — 24 | — 65 | — 79 | — 34 | — 33 |
| Uricase | — 60 | — 49 | — 11 | — 15 | — 74 | — 71 | — 34 | — 29 |
| Xanthine oxidase | — 15 [4] | — 88 [4] | — 82 | — 77 | — 44 [4] | — 93 [4] | — 85 | — 81 |
| Pyruvate oxidation | | — 10 | | — 4 | | | | |

[1] Per milligram of nitrogen.
[2] Per liver.
[3] Interpreted as no change.
[4] Both were markedly reduced.

pared to the ad libitum controls. It can be concluded that the restriction in the food intake decreases the total amount of enzyme in the livers of both the depleted and pair-fed animals, but only because the livers are smaller, and that the absence of protein from the diet has no further effect.

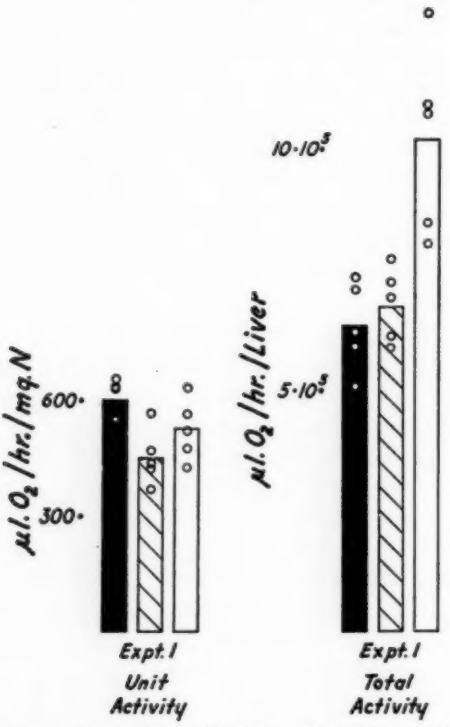


Fig. 1 Cytochrome oxidase activity. Black bar, depleted animals; hatched bar, pair-fed animals; white bar, ad libitum-fed animals.

The marked decrease in liver protein that occurs due to the absence of dietary protein does not involve the cytochrome oxidase protein.

The results obtained with the succinoxidase assay in experiment 1 are presented in figure 2 and summarized in

table 3. Both the unit activity and the total activity were markedly reduced. These changes are in agreement with the results obtained in two preliminary studies, where the depletion reduced the unit activity by 61 and 65%, respectively; there were no ad libitum-fed controls. It has been claimed

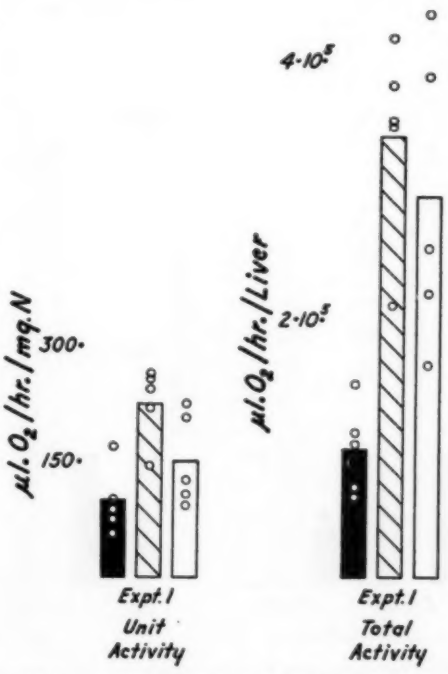


Fig. 2 Succinoxidase activity. Black bar, depleted animals; hatched bar, pair-fed animals; white bar, ad libitum-fed animals.

that the monometric assay for succinoxidase activity in the presence of excess cytochrome *c* is a measure of succinic dehydrogenase activity because the succinic dehydrogenase is rate-limiting. However, since this has not been demonstrated for protein-depleted animals, it seems safer to continue to term this the "succinoxidase assay."

Since it seemed likely at the time of the preliminary experiments with the succinoxidase system that the change could be attributed to a decrease in the succinic dehydrogenase, the levels of this enzyme were assayed in the two experiments. In both instances there was a marked decrease

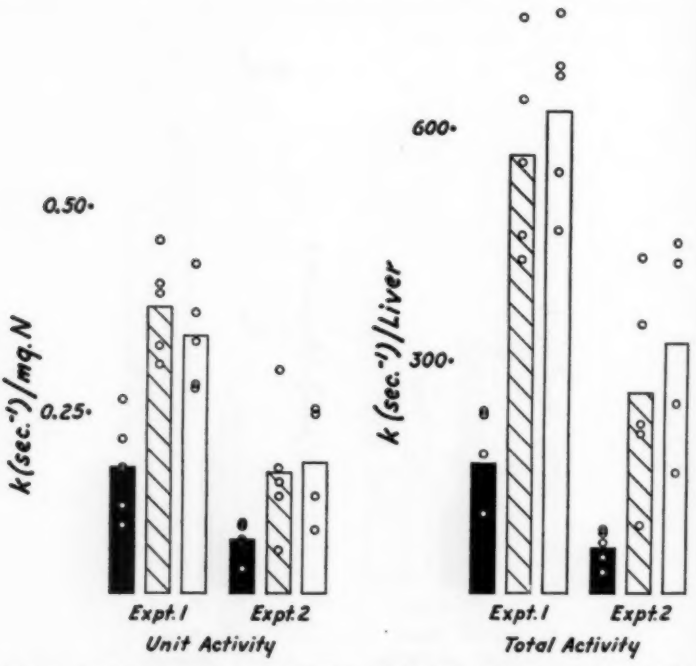


Fig. 3 Succinic dehydrogenase activity. Black bar, depleted animals; hatched bar, pair-fed animals; white bar, ad libitum-fed animals.

in the unit activity and the total activity when the depleted animals were compared to the pair-fed animals (fig. 3 and table 3). There was no significant change in either the unit or the total activity when the pair-fed were compared to the ad libitum animals. A comparison of the percentage decrease in unit activity (depleted vs. pair-fed) for the succinic dehydrogenase (58%) and for the succinoxidase (61%)

in experiment 1 suggests that the change can be attributed to the decrease in the succinic dehydrogenase.

D-Amino acid oxidase (fig. 4), DPN-cytochrome *c* reductase (fig. 5), and uricase (fig. 6), as is summarized in table 3, all

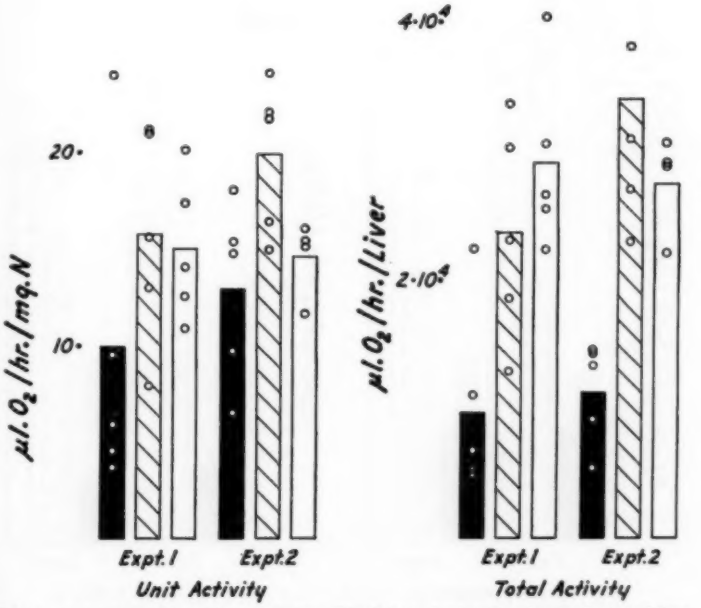


Fig. 4 D-Amino acid oxidase activity. Black bar, depleted animals; hatched bar, pair-fed animals; white bar, ad libitum-fed animals.

decreased in unit activity and total activity when the depleted animals and the pair-fed controls were compared.

The results with xanthine oxidase were in marked contrast to the results with all of the other enzymes (fig. 7 and table 3). Food restriction (pair-fed vs. ad libitum) reduced both the unit activity and the total activity to such an extent that

the effects of the protein depletion were not evident in experiment 1 (depleted vs. pair-fed) and were not significant in experiment 2, although a larger series might have shown a difference. The results are in agreement with those of

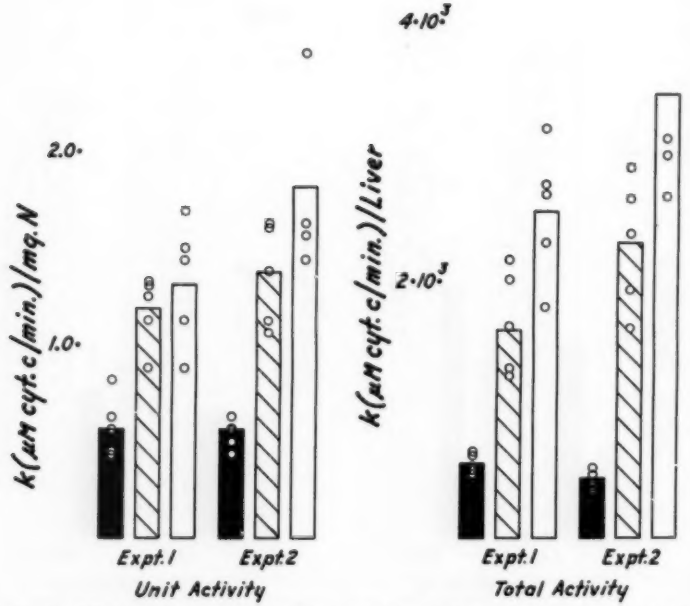


Fig. 5 DPN-cytochrome *c* reductase activity. Black bar, depleted animals; hatched bar, pair-fed animals; white bar, ad libitum-fed animals.

the numerous investigators who have reported that xanthine oxidase is an extremely labile enzyme, not only in protein depletion but in inanition as well.

In the one experiment with pyruvate oxidation (fig. 8 and table 3) it was found that depletion and restriction had no effect on the unit activity of the liver residue preparation.

The same was true when fumarate was not added as an accessory factor or when fumarate alone served as the substrate.

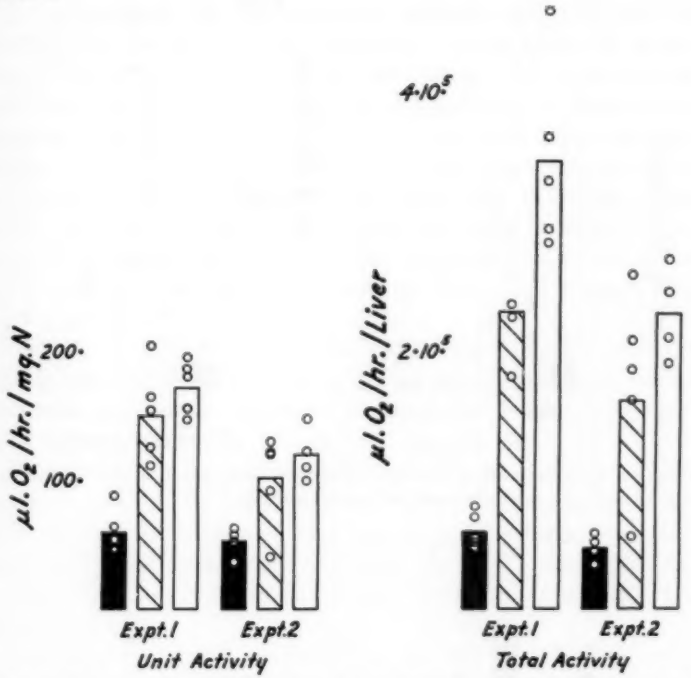


Fig. 6 Uricase activity. Black bar, depleted animals; hatched bar, pair-fed animals; white bar, ad libitum-fed animals.

## SUMMARY

The activities of the following oxidative enzymes or enzyme systems of rat liver have been studied after the experimental animals spent 49 to 53 days on a protein-free diet accompanied by sub-acute food restriction: cytochrome oxidase, succinoxidase, succinic dehydrogenase, D-amino acid oxidase, DPN-cytochrome *c* reductase, uricase, xanthine oxidase and pyruvate oxidation. Pair-fed animals and ad libitum-fed animals served as controls.

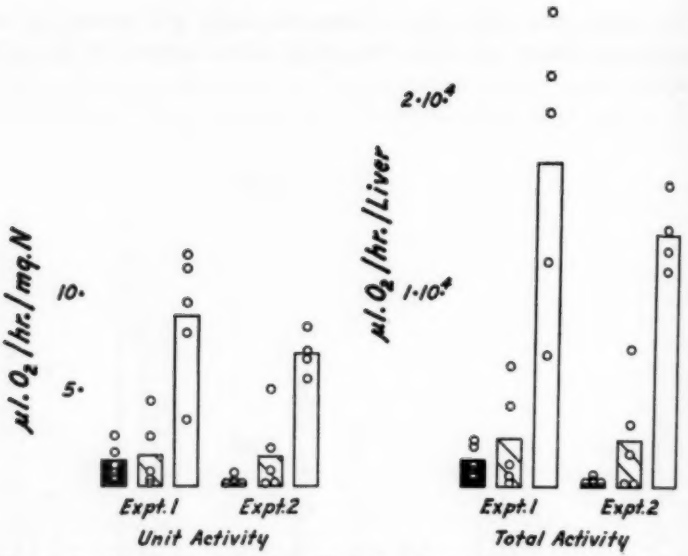


Fig. 7 Xanthine oxidase activity. Black bar, depleted animals; hatched bar, pair-fed animals; white bar, ad libitum-fed animals.

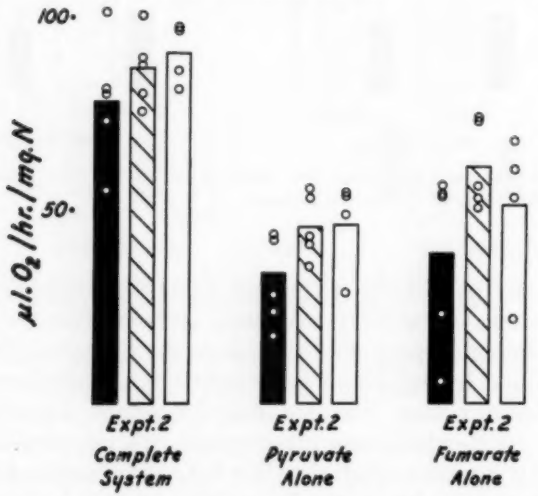


Fig. 8 Pyruvate oxidation. Black bar, depleted animals; hatched bar, pair-fed animals; white bar, ad libitum-fed animals.

The results have been expressed on the basis of the activity per milligram of nitrogen (the unit activity) and the activity per liver (the total activity).

A comparison of the depleted animals and the pair-fed controls on the basis of unit activity reveals that the activities of the enzyme systems decrease under the experimental conditions employed, except that the activity of cytochrome oxidase increases, and xanthine oxidase and pyruvate oxidation by the residue preparation show no change. The xanthine oxidase activities were markedly reduced in the livers of both groups. The general decrease in activity per unit of nitrogen indicates that the enzyme proteins were lost more rapidly than was the total protein. Cytochrome oxidase protein was conserved.

Sub-acute food restriction (a comparison of pair-fed controls and ad libitum-fed controls) had no effect on unit activities, except that in the case of xanthine oxidase the unit activity was markedly reduced.

Protein depletion decreases the total liver activity of 5 of these systems. The total cytochrome oxidase activity is not affected, and the total xanthine oxidase activity is so markedly reduced by the small restriction in the food intake that the further omission of all of the dietary protein has no apparent significant effect.

The food restriction suffered by the pair-fed animals, with the ad libitum-fed animals as controls, decreased the total activities of 4 enzymes (cytochrome oxidase, DPN-cytochrome *c* reductase, uricase and xanthine oxidase). The total succinoxidase activity was unchanged. The differences for succinic dehydrogenase and D-amino acid oxidase are not considered significant in spite of the fact that the livers of the restricted rats contained significantly less protein than those of the controls.

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# VITAMIN $B_{12}$ AND FOLIC ACID IN MILK DIETS[1]

R. A. COLLINS,[2] M. SCHREIBER, C. A. ELVEHJEM AND E. B. HART
*Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*

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Hart, Steenbock, Waddell and Elvehjem ('28) observed that the occurrence of anemia in rats fed a cow's milk diet could be prevented by the addition of iron and copper. Optimum growth was finally obtained by Kemmerer, Elvehjem, Hart and Fargo ('32) when traces of manganese were also added to cow's milk.

In spite of the discovery that iron and copper could cure cow's milk anemia, a number of European workers reported the failure of these minerals to cure anemia produced in rats and infants as a result of goat's milk feeding. Since liver, liver extracts, and yeast were found to cure this condition, Rominger and Bomskov ('33), Rominger, Meyer and Bomskov ('33), and György ('34) suggested that goat's milk may be deficient in Castle's "extrinsic factor." Studies conducted in the United States demonstrated no essential difference between cow's milk and goat's milk with respect to hemoglobin formation when iron and copper were added to milk diets fed to rats (Beard and Boggess, '35; Kohler, Elvehjem and Hart, '35; Orten and Smith, '36). However, when manganese was included in the mineral supplement, Kohler, Elvehjem and Hart ('35) observed that the growth of rats on goat's milk was inferior to that on cow's milk. The supplementation of this diet with brain tissue was found to produce normal growth. Also

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[2] Present address: International Minerals and Chemical Corporation, Central Research Laboratory, Skokie, Illinois.

the nutritive value of goat's milk was thought to have been improved by feeding the goats a better grade of alfalfa hay.

Since the growth aspect of this problem had not been resolved, the possibility of a folic acid or a vitamin $B_{12}$ deficiency, or both, in the goat's milk diet was investigated. The preliminary report by Collins, Dietrich and Elvehjem ('50) indicated that this hypothesis was correct. Collins, Harper, Schreiber and Elvehjem ('51) have found goat's milk and cow's milk to contain a comparable level of folic acid (1 to 4 μg per liter); however, the vitamin $B_{12}$ content of goat's milk was found to be 0.15 μg per liter, while a much higher level of 3 to 4 μg per liter was observed in cow's milk. Studies with the rat confirmed these analytical values and in addition demonstrated a relationship between the vitamin $B_{12}$ and folic acid requirement.

## EXPERIMENTAL

Male weanling rats, weighting 40 to 45 gm, of the Sprague-Dawley strain were purchased from a local animal farm. No attempt was made to secure weanlings having low body stores of vitamin $B_{12}$ or folic acid. The rats were housed in raised screen-bottom cages in the air-conditioned animal room of this laboratory. The milk diets were fed ad libitum each morning and evening to prevent souring. The milk was mineralized by adding 3 mg of iron, 0.15 mg of copper, and 0.15 mg of manganese per 100 ml of milk, using ferric pyrophosphate, copper sulphate and manganese sulphate, respectively. The goat's milk used in this study was obtained daily from the University dairy goat herd. The goats were kept in a dry lot and were fed a ration consisting of a grain mixture, corn silage and alfalfa hay. The raw cow's milk was obtained from market cow's milk of the University Creamery.

The vitamin $B_{12}$ and folic acid contents of the milk fed in this study were determined microbiologically using the procedure described by Collins et al. ('51). The folic acid and the vitamin $B_{12}$ contents of the rat liver were determined microbiologically using *Streptococcus faecalis* R and *Lactobacillus*

*leichmannii* (American Type Culture Collection 4797) as the test organisms. The folic acid activity of the rat liver was liberated during a 24-hour autolysis period at pH 7.0 and was measured using the medium of Luckey et al. ('45). The vitamin $B_{12}$ activity was released during a 24-hour tryptic digestion period at pH 8.0 and was determined using the medium of Thompson et al. ('50).

## RESULTS

### *Normal weanling rat studies*

The inferior nutritive value of mineralized goat's milk as compared to mineralized cow's milk may be seen in table 1, groups 1 and 11. The addition of 50 μg of folic acid or 0.1 μg of vitamin $B_{12}$ per rat per day to mineralized goat's milk resulted in similar but subnormal growth responses (groups 2 and 3); however, when these vitamins were added together and fed to group 4, a growth response equal to that of the cow's milk-fed animals of group 11 resulted. Also the addition of a higher level of vitamin $B_{12}$, 0.5 μg per day to the milk fed group 5, and the supplementation of the milk fed group 6 with 1.0 gm of fresh beef liver, resulted in maximum weight gains. The supplementation of group 7 with 0.1 gm of fresh beef liver (equivalent to 0.1 μg of vitamin $B_{12}$ activity) and the addition of 0.1 U.S.P. unit of liver extract (equivalent to 0.1 μg of vitamin $B_{12}$) to the milk fed group 8 were observed to result in a growth response similar to that obtained with the 0.1 μg of crystalline vitamin $B_{12}$ given group 3. The intraperitoneal injection of the animals in group 9 with folic acid was also found to stimulate growth. The growth response produced by grass juice supplementation (group 10) may have been due to its folic acid content.

The folic acid level of the liver tissue of the rats which received goat's milk was increased when folic acid was administered; however, a higher level was observed when folic acid was fed in conjunction with added vitamin $B_{12}$, group 4, and with the naturally occurring vitamin $B_{12}$ of cow's milk, groups

TABLE 1

*The influence of folic acid, vitamin $B_{12}$, beef liver, liver extract and grass juice upon normal weanling rats fed mineralized goat's and cow's milk diets*

| ADDITION/RAT/DAY | WEIGHT GAIN | | LIVER VITAMIN (Average of 6 rats) | | HEMOGLOBIN (Average of 6 rats) | | |
|---|---|---|---|---|---|---|---|
| | 5 weeks | No. of rats | Folic acid | Vitamin $B_{12}$ | 0 weeks | 3 weeks | 5 weeks |
| | *gm* | | *μg/gm* | *μg/mg* | *gm* | *gm* | *gm* |
| Goat's milk | | | | | | | |
| 1. None | 124 ± 4.0 [1] | 22 (3) [2] | 0.5 ± 0.1 [1] | 0.01 ± 0.003 [1] | ... | 12.3 | 13.1 |
| 2. 50 μg folic acid | 145 ± 4.0 | 20 (3) | 1.8 ± 0.2 | 0.01 ± 0.003 | .. | 12.4 | 13.7 |
| 3. 0.1 μg vitamin $B_{12}$ | 151 ± 6.6 | 22 (3) | 1.1 ± 0.5 | 0.02 ± 0.002 | .. | 11.2 | 13.2 |
| 4. 50 μg folic acid + 0.1 μg vitamin $B_{12}$ | 172 ± 4.1 | 22 (3) | 4.6 ± 0.4 | 0.03 ± 0.005 | .. | 12.5 | 14.7 |
| 5. 0.5 μg vitamin $B_{12}$ | 173 ± 6.2 | 9 (2) | | | | | |
| 6. 1.0 gm fresh beef liver | 174 ± 3.7 | 9 (2) | | | | | |
| 7. 0.1 gm fresh beef liver (≅ 0.1 μg vitamin $B_{12}$) | 162 ± 7.3 | 6 (1) | | | | | |
| 8. 0.1 U.S.P. unit liver extract (≅ 0.1 μg vitamin $B_{12}$) | 158 ± 5.6 | 6 (1) | | | | | |
| 9. 50 μg folic acid (injected) | 157 ± 2.0 | 6 (1) | | | | | |
| 10. 3 ml grass juice [3] | 159 ± 5.1 | 6 (1) | | | | | |
| Cow's milk | | | | | | | |
| 11. None | 178 ± 5.4 | 12 (2) | 0.4 ± 0.1 | 0.04 ± 0.005 | 8.9 | 11.9 | 13.9 |
| 12. 50 μg folic acid | 178 ± 8.8 | 6 (1) | 3.0 ± 0.6 | 0.05 ± 0.003 | 9.4 | 12.5 | 13.4 |
| 13. 0.1 μg vitamin $B_{12}$ | 173 ± 10.1 | 6 (1) | 0.7 ± 0.2 | 0.06 ± 0.006 | 10.1 | 12.1 | 14.5 |
| 14. 50 μg folic acid + 0.1 μg vitamin $B_{12}$ | 182 ± 10.5 | 6 (1) | 4.2 ± 0.6 | 0.06 ± 0.007 | 9.0 | 12.0 | 14.9 |

[1] Standard error of the mean.

[2] Number of experiments.

[3] Pressed from lawn clippings and found to contain 7 μg of free folic acid activity per milliliter.

12 and 14. The liver content of vitamin $B_{12}$ appeared to be correlated with growth, whereas the folic acid content of the liver did not. An initial experiment indicated that the addition of vitamin C to mineralized cow's milk resulted in an increase in the liver vitamin $B_{12}$ level (Collins, Dietrich and Elvehjem, '50); however, further work has failed to confirm this observation.

The hemoglobin level of the normal weanling rats referred to in table 1 increased slowly from 9 or 10 gm at weanling age to the normal level of 14 gm after 5 weeks, and was not observed to be influenced by the addition of folic acid or vitamin $B_{12}$ to mineralized goat's milk or cow's milk diets. The hemoglobin values for the rats fed the goat's milk diets at zero weeks (groups 1, 2, 3 and 4) may be considered similar to those of the animals fed cow's milk (groups 11, 12, 13 and 14), since this phase of the experiment was conducted concurrently from the same shipment of weanling rats.

The growth-promoting activity of 0.5 μg of vitamin $B_{12}$ when added to goat's milk and the low folic acid level of the liver of rats fed goat's milk and cow's milk strongly suggest that the lower nutritive value of goat's milk is due primarily to its low content of vitamin $B_{12}$. Thus, when 3 μg of vitamin $B_{12}$ were added per liter of goat's milk, growth equal to that obtained with a cow's milk diet was attained (table 2, group 3). The addition of 500 μg of folic acid per liter to the goat's milk fed group 2 resulted in highly variable growth.

### *Anemic rat studies*

The influence of folic acid and vitamin $B_{12}$ upon the rate of hemoglobin regeneration and growth, using anemic rats, is presented in table 3. Weanling rats were made anemic by feeding non-mineralized goat's milk or cow's milk for a period of 4 weeks. At this time the severely anemic rats were fed their respective mineralized milk diets with added folic acid and vitamin $B_{12}$. The inferior growth of rats fed the mineralized goat's milk diet was again improved by the addition of vitamin $B_{12}$, while the growth of animals receiving the mineralized

TABLE 2

*Rat growth using mineralized milk diets*

| TREATMENT | ADDITION PER LITER OF MILK | NO. OF RATS | WEIGHT GAIN IN 5 WEEKS |
|---|---|---|---|
| | μg | | gm |
| Goat's milk [1] | | | |
| 1. None | ... | 8 | 126 ± 9.6 [3] |
| 2. Folic acid | 500 | 8 | 139 ± 11.1 |
| 3. Vitamin $B_{12}$ | 3.0 | 8 | 158 ± 3.9 |
| 4. Folic acid + vitamin $B_{12}$ | 500 + 3.0 | 8 | 158 ± 4.8 |
| Cow's milk [2] | | | |
| 5. None | ... | 7 | 159 ± 6.5 |
| 6. Folic acid | 500 | 8 | 168 ± 5.3 |

[1] Average folic acid and vitamin $B_{12}$ contents were found to be 0.25 μg and 0.16 μg per liter.

[2] Average folic acid and vitamin $B_{12}$ contents were found to be 2.4 μg and 3.8 μg per liter.

[3] Standard error of the mean.

TABLE 3

*The response of anemic rats to mineralized goat's milk and cow's milk diets with added folic acid and vitamin $B_{12}$*

(6 rats per group)

| ADDITION/RAT/DAY | GOAT'S MILK-FED RATS [1] | | COW'S MILK-FED RATS [2] | |
|---|---|---|---|---|
| | Wt. gain in 4 weeks | Hemoglobin 1st week | Wt. gain in 4 weeks | Hemoglobin 1st week |
| | gm | gm | gm | gm |
| 1. None | 68 ± 5.3 [3] | 12.0 | 107 ± 9.9 [3] | 10.6 |
| 2. 50 μg folic acid | 54 ± 16.7 | 11.0 | 118 ± 4.7 | 10.1 |
| 3. 0.1 μg vitamin $B_{12}$ | 92 ± 8.3 | 12.0 | 111 ± 7.5 | 8.2 |
| 4. 50 μg folic acid + 0.1 μg vitamin $B_{12}$ | 99 ± 10.8 | 12.7 | 124 ± 5.8 | 9.6 |

[1] Average initial hemoglobin level, 6.0 gm.
Average initial weight, 93 gm.

[2] Average initial hemoglobin level, 4.3 gm.
Average initial weight, 107 gm.

[3] Standard error of the mean.

cow's milk diet was not influenced by the addition of vitamin $B_{12}$. The rate of hemoglobin regeneration was rapid when these mineralized milks were fed and was not influenced by the vitamin additions.

### *Scaly dermatosis*

During the course of this work a dry, scaly dermatosis was observed on the paws of the weanling rats which were fed the goat's milk and cow's milk diets. Further studies by Collins, Schreiber and Elvehjem ('51) and by Schreiber, Collins, Niño-Herrera and Elvehjem ('52) have implicated lactose as an essential dietary component for the production of this dermatosis. The histological aspect of this dermatosis has been studied by Niño-Herrera et al. ('53).

## DISCUSSION

Although 0.1 μg of vitamin $B_{12}$ per rat per day was initially thought to be sufficient to meet the requirement of the rat for this vitamin, a higher level appears to be required for maximum growth when a mineralized goat's milk diet is fed. The high vitamin $B_{12}$ requirement of the rat receiving a goat's milk diet, in contrast to a lower requirement by animals receiving other types of diets, may be related to the presence of the carbohydrate, lactose. Hartman, Dryden and Cary ('49) have observed that the growth of factor X-deficient rats was decreased further by the addition of lactose to the factor X-deficient diet, and that this growth depression could be reversed by vitamin $B_{12}$.

The ability of folic acid to spare a part of the vitamin $B_{12}$ requirement of rats fed a mineralized goat's milk diet is seen in table 1, groups 2, 3 and 4. Folic acid: (1) failed to replace vitamin $B_{12}$ completely (table 1, group 2); (2) produced extremely variable growth (table 2, group 2); and (3) failed to produce a growth response in rats previously made anemic by a goat's milk diet (table 3, group 2). The extent to which folic

acid spares vitamin $B_{12}$ may depend upon the vitamin $B_{12}$ stores of the animals.

In confirmation of the work of Kohler, Elvehjem and Hart ('35), mineralized goat's milk was found adequate for hemoglobin formation. The relationship of age to the rate of hemoglobin formation, using both goat's milk and cow's milk diets, was observed to be similar to the results obtained by Elvehjem, Hart and Sherman ('33). The hemoglobin level in normal weanling rats increased slowly, whereas the level in older, severely anemic rats increased very rapidly when iron and copper were supplied. Since the vitamin $B_{12}$-deficient goat's milk diet did not limit hemoglobin formation, the requirement for hemoglobin synthesis appears to be very low in comparison to the amount required for growth.

The postulations by Rominger, Meyer and Bomskov ('33) and György ('34) that goat's milk may be deficient in Castle's "extrinsic factor" have been verified. Since no clinical evidence is available, it is not possible at this time to estimate the extent to which the low vitamin $B_{12}$ content of goat's milk may contribute to "goat's milk anemia" in infants.

## SUMMARY

The inferior growth of rats fed a mineralized goat's milk diet was found to be the result of a vitamin $B_{12}$ deficiency. Rat growth equal to that obtained with a cow's milk diet was attained when 0.5 μg of vitamin $B_{12}$ was added per rat per day to a goat's milk diet, or when 3 μg of vitamin $B_{12}$ were added per liter of goat's milk.

Folic acid spared a part of the vitamin $B_{12}$ requirement of the rat fed a mineralized goat's milk diet.

Folic acid and vitamin $B_{12}$ additions to mineralized goat's milk or cow's milk diets did not influence the rate of hemoglobin formation in the normal or the anemic rat.

A dry, scaly dermatosis was observed upon the paws of weanling rats fed a goat's milk or a cow's milk diet.

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# THE EFFECTS OF VITAMINS $B_{12}$ AND $B_{12f}$ ON GROWTH, KIDNEY HEMORRHAGE, AND LIVER FAT IN RATS FED PURIFIED DIETS[1]

EDGAR A. HAWK[2] AND C. A. ELVEHJEM
*Department of Biochemistry, University of Wisconsin, Madison*

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## INTRODUCTION

Vitamin $B_{12}$ is known to protect the young rat on a diet low in choline and methionine against renal hemorrhage and fatty liver (Schaefer et al., '50; Strength et al., '51). Under other conditions, vitamin $B_{12}$ protects against fatty liver in the complete absence of a dietary intake of labile methyl groups (Bennett et al., '51). The new and biologically different form of vitamin $B_{12}$, tentatively assigned the designation $B_{12f}$, recently reported by Lewis et al. ('52a) exhibits a high degree of activity for *Lactobacillus leichmannii, Escherichia coli,* and *Euglena gracilis,* but is completely inactive in the rat growth assay. Although growth-stimulating activity for the chick was reported, a more highly purified preparation was later found to be inactive in this species (Lewis et al., '52b).

We wish to report that the new vitamin has no choline-sparing activity in young rats fed purified diets. In the course of this work, effects of variations in dietary cystine,

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methionine, and protein have been observed which we also wish to report in this paper.

## METHODS

The composition of the basal diet used in all experiments except as otherwise specified is given in table 1. Although a preliminary experiment indicated that under the conditions used vitamin $B_{12}$ by mouth is effective, but less so than when given parenterally, both vitamins $B_{12}$ and $B_{12f}$ were given by intraperitoneal injection in all of the work reported herein.

TABLE 1

*Composition of basal diet*

| CONSTITUENTS | AMOUNT |
|---|---|
| | % |
| Casein [1] | 9.0 |
| L-cystine | 0.3 |
| Sucrose | 81.0 |
| Corn oil | 5.0 |
| Salts IV [2] | 4.0 |
| Vitamin mixture [3] | 1.0 |

[1] Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Salts IV; Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., J. Biol. Chem., 1941, *138:* 459.

[3] Containing, in milligrams per gram: thiamine hydrochloride 0.3, riboflavin 0.3, niacin 2.0, pyridoxine hydrochloride 0.2, calcium pantothenate 2.0, folic acid 0.025, biotin 0.01, inositol 10.0, and *p*-aminobenzoic acid 12.5, in sucrose.

Oral administration would appear to have an advantage in nutritional experiments, but it was felt that the parental route was preferable because of constancy and certainty of dosage. In addition, Lewis et al. ('52b) have shown that vitamin $B_{12f}$ is very poorly, if at all, absorbed from the gastrointestinal tract, and only injection of the substance would insure the opportunity for it to act in the tissues. The vitamin $B_{12}$ solution was made from mannitol triturate,[3] and the vitamin $B_{12f}$ preparation was diluted from a highly purified concentrate which had been assayed microbiologi-

[3] Merck's.

cally by the method of Thompson et al. ('50), using *Lactobacillus leichmannii* as the test organism. All concentrations were adjusted so that the daily dose was contained in 0.1 ml. Control animals were injected with water.

The animals were weanling males of the Sprague-Dawley strain (age 21 days, weight range 30 to 52 gm). On arrival at the laboratory, each lot was divided into groups of similar mean weight, including in all cases appropriate control groups given diets containing the levels of choline specified in the tables. Each rat was given one drop of haliver oil when placed on experiment and one drop a week later. An experimental period of 10 days was selected in order to sacrifice the animals at a time when the incidence of kidney damage would be near the peak. During this period the animals were housed in an air-conditioned room in wide-mesh screen-bottom individual cages and were given access to food and water ad libitum.

At the end of the 10-day period, all surviving animals were sacrificed by decapitation and the kidneys and livers removed. The kidneys were weighed in pairs and the degree of hemorrhage rated on an arbitrary scale ranging from zero for grossly normal to 4 + for severe hemorrhage with no apparent normal tissue. The livers were weighed, dried in an oven at approximately 90°C., weighed again, and the fat content determined as total ether-extractable material. Data are not presented for kidney weight, which was closely correlated with degree of visible hemorrhage, or for per cent liver water, which varied inversely with the amount of fat present. Animals that died before the termination of the experimental period were autopsied within a few hours, and the data for their kidney and liver findings are included in the tables. However, the growth figures represent only those animals that survived the full 10-day period.

## RESULTS

Table 2 summarizes the combined results obtained in the first series of experiments, using animals fed the basal 9%

casein diet. Control animals not receiving choline in the diet developed a severe deficiency characterized by very poor growth, marked kidney hemorrhage, and fatty livers, with only 6 out of 10 animals' surviving the full 10-day experimental period. Five-tenths of a microgram vitamin $B_{12}$/day permitted complete survival, growth approximately

TABLE 2

*Effects of vitamins $B_{12}$ and $B_{12f}$ in animals fed the 9% casein basal diet*

| DIETARY CHOLINE CHLORIDE | DAILY SUPPLEMENT | TOTAL NO. ANIMALS | SURVIVORS | | ALL ANIMALS | |
|---|---|---|---|---|---|---|
| | | | No. | Growth/ 10 days | Kidney hemorrhage [1] | Liver fat, % fresh weight |
| % | | | | gm | | |
| 0.1 | 0 | 30 | 29 [2] | 16 ± 0.5 [3] | 0 | 8.0 ± 0.2 [3] |
| 0.1 | 0.5 μg vitamin $B_{12}$ | 5 | 5 | 21 ± 0.8 | 0 | 7.4 ± 0.3 |
| 0 | 0 | 10 | 6 | 3 ± 1.3 | 3.9 (10) [4] | 20.1 ± 1.2 |
| 0 | 0.1 μg vitamin $B_{12}$ | 10 | 10 | 11 ± 1.4 | 1.7 (8) | 24.0 ± 1.6 |
| 0 | 0.5 μg vitamin $B_{12}$ | 10 | 10 | 17 ± 0.9 | 0.2 (1) | 26.0 ± 0.9 |
| 0 | 0.1 μg vitamin $B_{12f}$ | 10 | 5 | 8 ± 1.4 | 3.4 (9) | 22.6 ± 1.6 |
| 0 | 0.5 μg vitamin $B_{12f}$ | 10 | 6 | 7 ± 2.0 | 3.3 (9) | 21.2 ± 1.5 |
| 0.02 | 0 | 15 | 15 | 13 ± 0.9 | 0.6 (5) | 21.5 ± 1.2 |
| 0.02 | 0.5 μg vitamin $B_{12}$ | 15 | 15 | 16 ± 1.2 | 0 | 15.6 ± 1.0 |
| 0.02 | 0.5 μg vitamin $B_{12f}$ | 15 | 15 | 17 ± 1.4 | 1.1 (7) | 24.4 ± 1.2 |

[1] Based on an arbitrary scale of zero for no visible hemorrhage to 4 for marked hemorrhage with no visible normal renal tissue.

[2] One animal died from accidental trauma.

[3] Mean ± probable error.

[4] The number in parentheses indicates the total number of animals having hemorrhagic kidneys.

equal to that of the control animals receiving 0.1% choline chloride, and almost complete protection against renal hemorrhage. Survival was complete with 0.1 μg vitamin $B_{12}$/day, but growth was slower and protection of the kidneys incomplete. Liver fat levels tended to be higher in both groups of vitamin $B_{12}$-treated animals than in the controls receiving no choline, and the difference was statistically significant [4]

[4] Statistical significance of differences was determined according to the test procedure described in *Chemistry of Food and Nutrition*. Henry C. Sherman, 7th ed., The Macmillan Co., New York, N. Y., 1946.

in the case of animals receiving 0.5 μg vitamin $B_{12}$. In marked contrast, treatment with vitamin $B_{12f}$ at either of the two levels employed had little if any effect on survival, kidney hemorrhage, or liver fat. Although the survivors may have grown somewhat more than the controls receiving no choline, the variations within these groups were excessive and the differences were not significant. When 0.02% choline chloride was included in the diet, 0.5 μg vitamin $B_{12}$/day definitely lowered the level of liver fat, whereas the same amount of vitamin $B_{12f}$ had no such effect. With this suboptimum

TABLE 3

*Effects of vitamin $B_{12}$ and $B_{12f}$ in animals fed the 18% casein basal diet*

| DIETARY CHOLINE CHLORIDE | DAILY SUPPLEMENT | TOTAL NO. ANIMALS | SURVIVORS | | ALL ANIMALS | |
|---|---|---|---|---|---|---|
| | | | No. | Growth/ 10 days | Kidney hemorrhage | Liver fat, % fresh weight |
| % | | | | *gm* | | |
| 0.1 | 0 | 8 | 8 | 36 ± 1.3[1] | 0 | 3.3 ± 0.2[1] |
| 0.02 | 0 | 10 | 9 | 36 ± 1.9 | 0.9 (3)[2] | 16.4 ± 1.3 |
| 0.02 | 0.5 μg vitamin $B_{12}$ | 10 | 10 | 37 ± 1.6 | 0.4 (1) | 12.1 ± 0.5 |
| 0.02 | 0.5 μg vitamin $B_{12f}$ | 10 | 10 | 35 ± 1.3 | 1.0 (4) | 20.0 ± 0.9 |
| 0 | 0 | 5 | 5 | 21 ± 1.8 | 2.2 (5) | 20.8 ± 0.7 |
| 0 | 0.5 μg vitamin $B_{12}$ | 5 | 5 | 33 ± 2.2 | 0.8 (2) | 20.4 ± 1.3 |

[1] Mean ± probable error.

[2] The number in parentheses indicates the total number of animals having hemorrhagic kidneys.

level of dietary choline, neither vitamin $B_{12}$ nor $B_{12f}$ produced a significant change in growth rate.

When the basal diet was modified by increasing casein to 18% at the expense of sucrose, similar results were obtained (table 3). The groups receiving 0.02% choline chloride, or zero choline but with supplementary vitamin $B_{12}$, grew at approximately the same rate as did the 0.1% choline chloride controls. With no choline in the diet, all animals had visible kidney hemorrhage which was partially preventable by vitamin $B_{12}$. However, vitamin $B_{12}$ exhibited a lipotropic effect only when the suboptimum level of choline was included in the diet. Vitamin $B_{12f}$ definitely did not have a lipotropic

effect under the same conditions but actually tended to produce a higher level of liver fat.

Table 4 gives the effects of omitting cystine or adding methionine, gelatin, or casein to the basal 9% casein diet, with and without supplementary vitamin $B_{12}$. It will be

TABLE 4

*Effects of vitamin $B_{12}$ in animals fed various modifications of the 9% casein basal diet*

| DIETARY CHOLINE CHLORIDE | ADDITION TO BASAL DIET | DAILY SUPPLEMENT | TOTAL NO. ANIMALS | SURVIVORS | | ALL ANIMALS | |
|---|---|---|---|---|---|---|---|
| | | | | No. | Growth/10 days | Kidney hemorrhage | Liver fat, % fresh weight |
| % | | | | | gm | | |
| 0.1 | 0 | 0 | 15 | 15 | 17 ± 1.0 [2] | 0 | 7.5 ± 0.4 [2] |
| 0 | 0 | 0 | 15 | 9 | 11 ± 1.2 | 3.1 (13) [3] | 19.7 ± 1.0 |
| 0 | 0 [1] | 0 | 5 | 5 | 5 ± 0.7 | 0.2 (1) | 22.7 ± 1.7 |
| 0 | 0 [1] | 0.5 μg vitamin $B_{12}$ | 5 | 5 | 10 ± 0.7 | 0 | 19.7 ± 1.5 |
| 0 | 0.3% DL-methionine | 0 | 5 | 5 | 18 ± 0.3 | 0 | 15.1 ± 2.0 |
| 0 | 0.3% DL-methionine | 0.5 μg vitamin $B_{12}$ | 5 | 5 | 18 ± 0.8 | 0 | 12.3 ± 0.5 |
| 0 | 0.6% DL-methionine | 0 | 5 | 5 | 17 ± 0.4 | 0 | 15.7 ± 1.0 |
| 0 | 0.6% DL-methionine | 0.5 μg vitamin $B_{12}$ | 5 | 5 | 16 ± 0.9 | 0 | 14.5 ± 0.9 |
| 0 | 3% gelatin | 0 | 5 | 4 | 6 ± 1.8 | 2.4 (5) | 16.0 ± 1.4 |
| 0 | 3% gelatin | 0.5 μg vitamin $B_{12}$ | 5 | 5 | 21 ± 1.5 | 0.4 (2) | 28.9 ± 0.8 |
| 0 | 3% casein | 0 | 5 | 5 | 10 ± 1.6 | 2.8 (5) | 16.0 ± 2.0 |
| 0 | 3% casein | 0.5 μg vitamin $B_{12}$ | 5 | 4 | 27 ± 1.3 | 0.8 (1) | 22.7 ± 1.4 |

[1] Cystine omitted from basal diet.

[2] Mean ± probable error.

[3] The number in parentheses indicates the number of animals having hemorrhagic kidneys.

noted that the growth of the control animals receiving no choline was somewhat better than that of comparable animals listed in table 2. However, kidney hemorrhage was severe and the liver fat level approximately the same. Omission of cystine from the basal diet sharply reduced growth

and almost completely prevented kidney hemorrhage, but did not significantly change the level of liver fat. Vitamin $B_{12}$ had little effect except for a moderate growth stimulation. DL-Methionine fed at either of two levels permitted good growth considering the protein level of the diet and completely prevented kidney hemorrhage. The level of liver fat was reduced but definitely not down into the range for the controls fed 0.1% choline chloride. The effect of 0.3% methionine was approximately equal to that of 0.6% and vitamin $B_{12}$ had little or no additional effect.

The inclusion of 3% gelatin in the basal diet reduced the growth rate unless vitamin $B_{12}$ was given. In the latter case, the growth rate was even higher than in the control animals receiving 0.1% choline chloride, and liver fat was markedly increased. A similar stimulation in growth rate and a considerably less definite change in liver fat were observed when vitamin $B_{12}$ was given to animals receiving added casein (total 12%) instead of gelatin.

## DISCUSSION

Although vitamin $B_{12f}$ has chemical and physical properties similar to those of vitamin $B_{12}$ and in addition is highly active in microbiological assays, it appears to have little or no activity in higher animals, as judged by the tests thus far applied. However, under the conditions employed in the present work, vitamin $B_{12f}$ apparently did stimulate growth in an occasional animal. This effect is certainly not uniform and cannot be offered as evidence of widely applicable growth-stimulating ability.

Paper chromatography has revealed the presence of small amounts of vitamin $B_{12}$ in various preparations of vitamin $B_{12f}$ (Lewis et al., '52b). The vitamin $B_{12f}$ used in these studies was a highly purified concentrate which had been shown to contain only about 1% of its activity as vitamin $B_{12}$. Since 0.1 μg of vitamin $B_{12}$ gave suboptimum growth stimulation and did not completely protect kidneys when choline was omitted from the diet, it is believed that such

a minor degree of contamination with vitamin $B_{12}$ can be disregarded. Furthermore, the minute traces of vitamin $B_{12}$ which may be present in commercial "vitamin-free" casein have not interfered with the demonstration of activity even when casein was fed at the 18% level. It should be emphasized that the dosages of vitamin $B_{12f}$ shown in the tables represent only microbiological activity as measured by means of *Lactobacillus leichmannii,* using vitamin $B_{12}$ as a standard. Dosage was limited to 0.5 μg per rat per day because of the small supply of the substance available in purified form. The possibility remains that larger amounts of vitamin $B_{12f}$ might have some slight choline-sparing activity.

There is no doubt that vitamin $B_{12}$ spares choline, but the degree of sparing depends upon the criterion used for choline deficiency. When judged only on the basis of growth stimulation or kidney protection, dietary choline may not be needed when an adequate amount of vitamin $B_{12}$ is supplied. When the criterion is the level of liver fat, the lipotropic action of vitamin $B_{12}$ can be demonstrated only in the presence of suboptimum dietary choline. However, in no case was the level of liver fat in the animals fed a suboptimum level of choline with supplementary vitamin $B_{12}$ comparable with that in the controls fed 0.1% choline chloride. It is of interest to note that vitamin $B_{12}$ may actually increase the level of liver fat, possibly by promoting the synthesis of enough choline to satisfy the requirement for growth but not enough to promote the movement of fat out of the liver.

While it is well known that choline deficiency can be demonstrated best in growing animals, a severe limitation of growth caused by omission of cystine from or addition of gelatin to the diet did not prevent the development of fatty livers. On the other hand, with 0.1% choline chloride in the diet, a considerably lower level of liver fat was found in the faster growing animals receiving 18% casein than in those growing much more slowly on 9% casein. Thus it is possible that liver fat levels in all of our animals on the lower level of

protein may have been moderately increased because of protein deprivation, or because of a relative deficiency of some specific amino acid or other substance closely associated with protein. Under the conditions employed in these experiments, the effect of protein is apparently not due entirely to sulfur-containing amino acids. The finding that methionine will only partially replace choline confirms the work of Rose, Machella and György ('48), done with an amino acid diet. Even in the presence of vitamin $B_{12}$ and a presumably adequate supply of labile methyl groups, choline as such appears to be necessary in the diet. The growth differences obtained with and without vitamin $B_{12}$ on any of the various diets (without added choline) employed appear sufficient to suggest that the technique described above might be used as an assay method for vitamin $B_{12}$.

## SUMMARY

The choline-sparing activities of vitamins $B_{12}$ and $B_{12f}$ have been investigated, using weanling male rats fed purified diets containing 9% or 18% casein. As judged by growth stimulation and prevention of hemorrhagic kidneys, vitamin $B_{12}$ is able to replace dietary choline almost completely. On the other hand, a suboptimum level of dietary choline is necessary for the demonstration of lipotropic activity. No evidence was obtained of a choline-sparing activity of vitamin $B_{12f}$.

Under the conditions used, methionine will not replace choline completely even in the presence of supplementary vitamin $B_{12}$. Rapid growth is not essential for the development of fatty livers, although the higher levels of liver fat were usually found in faster growing animals. Even with 0.1% choline chloride in the diet, liver fat in animals fed the 9% casein diet was considerably higher than that found in animals fed the 18% casein diet, indicating that casein may have lipotropic activity in addition to that due to its methionine content.

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# EFFECT OF AUREOMYCIN ON NITROGEN AND WATER METABOLISM IN GROWING PIGS

R. BRAUDE[1] AND B. CONNOR JOHNSON
*Division of Animal Nutrition, University of Illinois, Urbana*

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It has been shown that under many conditions certain antibiotics, added in small amounts to food, will promote growth and improve the efficiency with which the animals utilize their food (Wallace, Braude and Cunha, '52; Braude and Wallace, '52). Two explanations of this action have been widely discussed, one based on a nutritional, the other on a disease approach. The present experiment was planned to provide some additional fundamental data concerning the effect which the feeding of an antibiotic has on the nitrogen metabolism of growing pigs and on their utilization of certain water-soluble vitamins. This report deals only with the nitrogen metabolism data, and with some observations made during the test concerning the water metabolism.

## EXPERIMENTAL

Nine litter-mate pairs of crossbred pigs were purchased from a commercial producer. The pigs were about 6 weeks old, of the same sex, and on arrival in the laboratory were all of about the same weight. They were well grown for their age, properly weaned and vaccinated against cholera. They were put into small individual metabolism cages, and were gradually introduced to the synthetic experimental diets. The composition of the diets is given in table 1. The only difference between the two diets was that one of them contained 20 gm per

[1] Smith Mundt and Fulbright Fellow from the National Institute for Research in Dairying, University of Reading, England.

TABLE 1

*Composition of the basal diet* [1]

| | % |
|---|---|
| Alpha-protein | 17.6 |
| DL-methionine | 0.4 |
| Cerelose | 66.0 |
| Lard | 10.0 |
| Mineral mixture | 6.0 |
| Vitamins | see below |

| MINERAL MIXTURE | % |
|---|---|
| $CaHPO_4$ | 33.80 |
| $K_2HPO_4$ | 25.80 |
| $CaCO_3$ | 22.90 |
| NaCl | 11.85 |
| $MgSO_4$ | 3.50 |
| Fe citrate | 1.91 |
| $MnSO_4$ | 0.10 |
| KI | 0.06 |
| $NaF_2$ | 0.03 |
| $CuSO_4\,5H_2O$ | 0.02 |
| $ZnCl_2$ | 0.02 |
| $CoCl_2$ | 0.01 |

| VITAMIN MIXTURE | *per kg* |
|---|---|
| Vitamin A | 2000 I.U. |
| Vitamin D | 200 I.U. |
| Vitamin E | 10 mg |
| Vitamin K | 100 μg |
| Thiamine | 10 mg |
| Riboflavin | 10 mg |
| Nicotinic acid | 30 mg |
| Pantothenic acid | 30 mg |
| Pyridoxine | 5 mg |
| Folic acid | 200 μg |
| Vitamin $B_{12}$ | 50 μg |
| Biotin | 10 μg |
| Choline | 1 gm |

[1] The experimental diet contained, as the only addition to the above, 20 gm of aureomycin per ton of diet.

ton of pure aureomycin. After mixing, the diets were made into pellets.[2] The alpha-protein diet was chosen because of previous experience indicating a good growth-promoting effect of antibiotics fed with such a diet (Wahlstrom, Cohn, Terrill and Johnson, '52; Nesheim and Johnson, '50). Pelleting was considered advisable in order to reduce wastage of food during the collection period. During the first two weeks the pigs were fed ad libitum, and their daily food and water intake was accurately recorded. Very soon after commencement of this

[2] The pelleting was carried out in the Akron, Ohio, plant of the Quaker Oats Company, through the courtesy of Dr. R. W. Graham. Their kind cooperation is gratefully acknowledged. The experimental meal mixtures were pelleted on a California Master Model Pellet Mill, through a 3/16" die. Although pressure and temperature figures are rough approximations, the pressure used was about 5 tons per square inch, and the temperatures employed ranged up to 125 to 135°F. Owing to the high fat content of the mix, steam was not used.

preliminary period it became obvious that both diets were causing severe diarrhea. In order to overcome this, methylated cellulose [3] was added at the rate of 4% of the ration. Such a treatment proved very successful, as with only a few exceptions normal consistency of feces was obtained. During the following three weeks the pigs were pair-fed, the initial amount of food being allocated on the basis of intake during the final stages of the preliminary period. The pair-feeding was successful only to a limited extent, as unfortunately during the time of the experiment extremely warm and humid weather prevailed and interfered with normal intake of food. The daily maximum temperatures varied between 81 and 99°F., with a relative humidity, recorded at 6:00 P.M., varying between 40 and 97%. All possible steps were taken to keep the animals comfortable in a room which was not air conditioned, but no doubt some of the animals were at times adversely affected. It should, however, be stressed that all the animals, whether on a diet with or without the antibiotic, were exposed to similar discomfort, and in the preliminary period were equally affected by the diarrhea mentioned above.

During the following two weeks all the animals were fed their respective diets ad libitum. Throughout the test, water was available to the pigs, and its daily intake was carefully recorded. During the last three days of each of the 5 weekly periods urine and feces collections were made. The urine was collected daily into brown glass bottles, and a small amount of hydrochloric acid was added to prevent decomposition. A composite three-day sample, 10% of each day's collection, was prepared. A portion of it was immediately analyzed for nitrogen, while the rest was stored under toluene in a refrigerator for vitamin estimations. Ferric oxide was fed as a marker at the beginning and end of each three-day period, and the feces voided between the markers were collected daily, dried in an oven at about 158°F., finely ground and analyzed for nitrogen.

[3] Methocel was kindly supplied by the Dow Chemical Company, Midland, Michigan, through the courtesy of Dr. Julius E. Johnson. The 4,000 CPS material was selected, since it has the greatest capacity for absorbing water.

On a few occasions, owing to intermittent scouring in a few of the pigs, samples of urine and feces were not collected. Only clear urine was sampled, and a few of the three-day averages are based on two collections only. A few of the feces data are missing, as only complete three-day samples were analyzed, and those from pigs scouring, even for brief periods only, could not be included.

## RESULTS

Before considering the results of this test, one should bear in mind the rather unfavorable conditions under which it was

TABLE 2

*Live weight gain, food and water intake and efficiency of food utilization*

| | CONTROL | EXPERMENTAL | |
|---|---|---|---|
| | Basal | Basal + aureomycin | |
| No. of pigs, paired litter mates | 6 | 6 | |
| Length of experimental period, days | 33 | 33 | |
| Average initial weight, kg | 15.95 | 16.37 | |
| Average live weight gain, kg | 11.05 | 12.19 | "t" = 2.15 |
| Average amount of food required for 1 kg gain, kg | 2.70 | 2.52 | "t" = 3.19[1] |
| Average daily food intake, gm | 899 | 924 | "t" = 0.43 |
| Average daily water intake, ml | 2,866 | 2,921 | "t" = 0.33 |
| Average food: water ratio (gm: ml) | 1: 3.13 | 1: 3.16 | |

[1] Significant at 0.05 level.

run. Owing to one death, to continuous scouring in one pair and lack of growth in another, only 6 pairs were taken into consideration when calculating the results. To satisfy any possible criticism of such a selection, it should be pointed out that when the data for all 9 pairs (until the death of one control pig during the 4th week of the test) are considered, all the mean values, except for rate of growth and food utilization, showed the same trend as those reported for the 6 pairs. Results for all 5 periods are treated together, as, owing to the circumstances mentioned above, we thought it unwise to consider the "paired feeding" periods separately from the ad

libitum feeding periods. Actually, feeding during the "paired" periods amounted to ad libitum feeding.

All the relevant data concerning growth, food and water intake and efficiency of food utilization are given in table 2. Data concerning urine and feces output and nitrogen and water metabolism are summarized in table 3.

The inclusion of aureomycin in the diet appeared to improve the growth rate in this test. The efficiency of food utilization was significantly better in the animals receiving the antibiotic.

TABLE 3

*Nitrogen and water metabolism*

| | CONTROL | | EXPERIMENTAL | | |
|---|---|---|---|---|---|
| | Basal | | Basal + aureomycin | | |
| No. of collection periods [1] | 5 | | 5 | | |
| Length of collection periods, days | 3 | | 3 | | |
| Average 3-day food intake, gm | 2,699 | (30) | 2,790 | (30) | "t" = 1.50 |
| Average 3-day feces output, gm | 223.4 | (19) | 228.8 | (24) | |
| Average feces output as a percentage of food intake | 8.3 | (19) | 8.2 | (24) | |
| Average 3-day water intake, ml | 9,017 | (30) | 9,181 | (30) | "t" = 1.09 |
| Average 3-day urine output, ml | 3,384 | (27) | 3,989 | (27) | "t" = 4.36 [2] |
| Average urine output as a percentage of water intake | 37.5 | | 43.1 | | "t" = 5.12 [2] |
| Average percentage N in the diet | 2.54 | | 2.54 | | |
| Average percentage N in the feces | 2.48 | (19) | 2.28 | (24) | |
| Average percentage N in urine | 0.94 | (27) | 0.86 | (27) | |
| Average 3-day intake of N, gm | 68.56 | (30) | 70.95 | (30) | "t" = 0.72 |
| Average 3-day intake of N of animals with complete excretion data, gm | 69.17 | (19) | 71.47 | (24) | |
| Average 3-day N excretion in feces, gm | 5.23 | (19) | 5.22 | (24) | |
| Average 3-day N excretion in urine, gm | 31.71 | (19) | 33.54 | (24) | |
| Average 3-day N retention, gm | 32.23 | (19) | 32.71 | (24) | |
| Average N retained as percentage of N intake | 46.6 | | 45.8 | | |

[1] Data for 6 animals in each group were collected. For the 5 collection periods all statistics reported should consist of averages for 30 readings. However, due to reasons stated in the text, the averages are based on the number of readings stated in parentheses.

[2] Significant at 0.001 level.

The pigs receiving the antibiotic consumed a little more food, and drank a little more water, but with the number of animals employed these differences were not statistically significant. The food: water intake ratio was the same for the two groups, indicating that if there was any trend for a higher water intake in the pigs receiving the antibiotic, it was due to higher food consumption rather than to any specific effect of the antibiotic.

It is evident that the addition of aureomycin to the diet had no effect on nitrogen utilization. All the recorded differences were small and not statistically significant.

The most striking result of this test concerns the water metabolism of the animals. The addition of an antibiotic to the diet caused a considerably higher (statistically highly significant) urine output. The difference could not be accounted for by the small increase in the water intake.

## DISCUSSION

The protein-sparing effect of antibiotics which has been reported by a number of investigators (Catron, Jensen, Homeyer, Maddock and Ashton, '52) cannot, on the basis of the evidence presented in this paper, be explained on the grounds of increased nitrogen retention caused by feeding a diet containing an antibiotic. This observation may possibly be explained by the fact that the requirement for protein, and particularly the protein: carbohydrate ratio, may not necessarily be the same in a fast-growing animal as in an animal growing at an apparently normal rate. Whatever is causing the antibiotic-receiving animals to grow faster may indirectly, by altering the optimum protein: carbohydrate ratio, be responsible for reducing the protein requirement of these animals.

The fact that faster-growing animals excreted considerably more urine, an amount of urine which cannot be accounted for by an increased water intake, than did their slower-growing litter mates kept under identical conditions, except for a small amount of an antibiotic added to the diet, offers a puzzle. Higher urinary excretion may in some circumstances indicate

a lower metabolic activity of the animal. Such lower activity would result in smaller respiratory losses and smaller loss of water through the skin, which in turn would cause a higher loss of water through the urine. Unfortunately, we have not collected data on the activity of our experimental animals, but it is conceivable that the feeding of an antibiotic may have lowered the metabolic rate of the animals. In some circumstances this by itself may lead to greater rate of fattening and improved food utilization. On the other hand, differential body and skin temperature, presuming a slightly elevated temperature of the control pigs, would explain the difference in urine volume and at the same time would be consistent with the antibiotic properties of aureomycin in suppressing gastrointestinal infection. Unfortunately, no such temperatures were taken during our test. The growth-promoting effect of an antibiotic may be due to its activity as a drug. A drug may cause a lowering of metabolic rate by creating a disturbance in the intestinal tract, a disturbance which could react on the activity of the animal. The extent of the disturbance may conceivably be influenced by the bacterial flora present in the intestinal tract, or rather by its specific reaction to the presence of the antibiotic in the intestinal contents.

## SUMMARY

Using a purified diet in the feeding of young pigs, the addition of aureomycin at the level of 20 mg/kg of ration brought about the expected improvement in rate of growth and increase in efficiency of food utilization. However, no effect on nitrogen retention was observed, even though there was an increase in efficiency of food utilization. Considerably higher urinary excretions (urinary water losses) were observed with animals receiving the antibiotic.

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# THE INFLUENCE OF THE NON-ESSENTIAL AMINO ACIDS ON THE REQUIREMENT OF THE ADULT RAT FOR ISOLEUCINE, METHIONINE AND THREONINE

MADELYN WOMACK, HARRIET A. HARLIN[1] AND PEI-HSING LIN[2]

*Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, United States Department of Agriculture, Washington, D. C.*

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The amount of methionine required for maximum growth in the young rat is reduced in the presence of cystine (Womack and Rose, '41) and similarly, the phenylalanine requirement is smaller if tyrosine is present in the ration (Womack and Rose, '46). No other interrelationship has been established in which the presence of a non-essential amino acid decreases the requirement of an essential one. In order to ascertain whether or not other such interrelationships exist, experiments were undertaken to determine the effect of: (1) cystine on the amount of methionine required to maintain adult rats in nitrogen equilibrium; (2) the non-essential amino acids other than cystine on the methionine requirement; and (3) the non-essential amino acids on the isoleucine and threonine requirement, respectively.

## EXPERIMENTAL

Adult male rats from the stock colony of this laboratory[3] varying in age from three to 6 months and in weight from 306

[1] Present address: Monsanto Chemical Company, Dayton, Ohio.

[2] Present address: Division of Animal Nutrition, University of Illinois, Urbana.

[3] A purebred strain resulting from the crossing of albino and black and white hooded strains of *Mus norvegicus*. The litters may include white, black, and black and white rats.

TABLE 1

*Composition of diets* [1]

| INGREDIENTS | DIET 1 [2] | DIET 2 [2] | VITAMINS ADDED PER KG RATION | AMOUNT |
|---|---|---|---|---|
| | *gm.* | *gm.* | | |
| L-histidine HCl·$H_2O$ | 0.27 | 0.27 | Thiamine HCl | 5 mg |
| DL-isoleucine | 0.40 | 0.40 | Riboflavin | 10 mg |
| L-leucine | 0.20 | 0.20 | Pyridoxine HCl | 5 mg |
| L-lysine HCl | 0.25 | 0.25 | Nicotinic acid | 5 mg |
| DL-methionine | 0.20 | 0.20 | Calcium *d*-pantothenate | 25 mg |
| DL-phenylalanine | 0.20 | 0.20 | *p*-Aminobenzoic acid | 300 mg |
| DL-threonine | 0.40 | 0.40 | α-Tocopherol acetate | 25 mg |
| DL-tryptophan | 0.20 | 0.20 | 2-Methyl-1,4-naphthoquinone | 2 mg |
| DL-valine | 0.40 | 0.40 | Biotin | 100 μg |
| DL-alanine | | 0.10 | Folic acid | 2 mg |
| L-arginine HCl | | 0.12 | | |
| L-aspartic acid | | 0.10 | | |
| L-cystine | | 0.10 | | |
| L-glutamic acid | | 0.10 | | |
| Glycine | | 0.10 | | |
| L-hydroxyproline | | 0.10 | | |
| L-proline | | 0.10 | | |
| DL-serine | | 0.10 | | |
| L-tyrosine | | 0.10 | | |
| Diammonium citrate | 1.15 | | | |
| Sodium bicarbonate | 0.22 | 0.27 | | |
| Sucrose | 76.76 | 76.84 | | |
| Salt mixture [3] | 4.00 | 4.00 | | |
| Corn oil (Mazola) | 3.00 | 3.00 | | |
| Lard | 12.00 | 12.00 | | |
| Vitamin A and D concentrate [4] | 0.05 | 0.05 | | |
| Inositol | 0.10 | 0.10 | | |
| Choline chloride | 0.20 | 0.20 | | |
| | 100.00 | 100.00 | | |

[1] The essential amino acids were incorporated in the diets at a level of 0.2% of the physiologically effective forms. The amounts listed in the table include the hydrochlorides, where used, and the unutilized D-forms.

[2] Experimental modifications: Diets 1A and 2A, methionine omitted; diet 1B, methionine omitted, 0.2% cystine added; diet 2B, methionine omitted, 0.2% cystine instead of 0.1% cystine added; diets 1C and 2C, isoleucine omitted; diets 1D and 2D, threonine omitted.

[3] Jones and Foster ('42).

[4] Squibb's Navitol, containing 65,000 U.S.P. units of vitamin A and 13,000 U.S.P. units of vitamin D per gram.

to 440 gm were the experimental subjects. In each experiment 6 animals were placed on either diet 1 or diet 2, the compositions of which are given in table 1.[4] One group of 6 was fed a diet containing 19 amino acids (diet 2). Litter mates of this group of approximately the same starting weight were fed the 9 amino acids essential for the maintenance of nitrogen balance in the adult rat (Wissler et al., '48) together with diammonium citrate (diet 1) in an amount sufficient to equalize the nitrogen content of the two rations.

The essential amino acids were fed at a level of 0.2% of the physiologically active form; that is, 0.4% where the D- forms are not utilized. These amounts were selected after preliminary trials in an attempt to formulate a ration which would contain low levels of amino acids but which would maintain adult animals in nitrogen equilibrium for subsequent studies; they bear no relationship to the requirements of the rat for individual amino acids or to the amino acid composition of well-utilized proteins. The non-essential amino acids, whether the L- or the DL- form, were fed at a level of 0.1% except in one study, where 0.2% cystine was fed. When an amino acid was omitted or fed at lower or higher levels than those mentioned above, the nitrogen content of the diet was not corrected; hence, the actual nitrogen contents of the different rations varied from 0.40 to 0.49% in the various experiments. The caloric value of the rations was calculated to be 4.4 (diet 1) and 4.6 (diet 2) calories per gram.

The animals were removed from the stock ration and placed directly upon the experimental low-nitrogen diets. Food and water were given ad libitum and weight changes and food consumptions were determined every 4 days. After a 20-day adjustment period on diets 1 or 2, during which individual nitrogen balances were determined for each 4-day period except the first, the animals were transferred to similar rations

[4] The amino acids were from various commercial sources. The purity of the DL-isoleucine and DL-threonine, both from Merck and Company, was checked by the microbiological method, and the L-leucine, from Interchemical Corp., was shown to contain no isoleucine. We wish to thank Dr. M. J. Horn and Mr. A. Blum of this laboratory for carrying out the assays.

from which a single essential amino acid had been omitted. They were kept on these diets for 20 days, and then the missing amino acid was supplied at various levels until the level was found which would maintain the animals in nitrogen equilibrium. Each level was fed for 20 days and each 20-day interval was divided into five 4-day periods for determination of nitrogen balances. If scattering of food occurred during any period, the data obtained during that period were omitted from consideration. The nitrogen balances for all the other periods for each level of amino acid feeding were averaged, since the adjustment to new levels was rapid enough to allow the inclusion of balances for the first with those for the later periods.

The animals were kept in an air-conditioned room with the temperature maintained at 80 $\pm$2°F. They were housed individually in wire metabolism cages with one-half inch mesh bottoms supported above 250-mm glass funnels. Under the funnels were glass bottles containing toluene and sufficient sulfuric acid to acidify the urine samples. A fine mesh screen was placed over the top of the funnel to retain the feces. During each period the feces were collected at intervals and stored until the end of the period either with or without the addition of sulfuric acid.[5] At the time of removal of the feces, the screens and funnels were washed with a fine stream of water and the washings retained for analysis. At the end of each period the combined urine samples and washings for each animal were filtered, the filter paper washed thoroughly, and filtrate and washings diluted to a convenient volume, usually 500 ml. The feces for each animal for each 4-day period were partially digested with sulfuric acid, mercury and potassium sulfate, cooled and quantitatively transferred to a volumetric flask. Aliquot portions were used for further digestion. Nitrogen was determined in the food, urine and feces by the Kjel-

[5] No differences were observed in the fecal nitrogen values obtained by the two methods of storing the feces. In one test during alternate periods the feces were removed daily and transferred to acid; during the other periods they were stored until the end of the period and then transferred to acid. The nitrogen determinations were made in the usual manner. Each set of 33 values averaged 46.1 mg fecal N/4 days.

dahl-Gunning-Arnold method (Association of Official Agricultural Chemists, '45) using mercury as a catalyst and distilling into boric acid (Sobel, Yuska and Cohen, '37). A two-hour digestion period was used for foods and 30 minutes for further digestion of the feces and for the urine samples. These periods of time were found to give maximum values. The Chibnall, Rees and Williams ('43) method gave no higher values and the shorter digestion periods represented a considerable saving in time from the 8-hour digestion period recommended by the latter authors.

## RESULTS AND DISCUSSION

The data obtained showing the effect of the presence or absence of the non-essential amino acids on the amount of methionine required to maintain adult rats in nitrogen equilibrium are summarized in table 2. That the absence of cystine is reflected in the nitrogen balances was apparent even as early as the first periods when methionine was omitted from the diets. The average daily nitrogen balance for the animals receiving no methionine and no cystine was — 35.2 mg and for those receiving 0.1% cystine but no methionine it was — 14.2 mg. Clearly, the presence of cystine decreases nitrogen losses on a methionine-free diet. DL-Methionine was added to the food until the amount needed to maintain nitrogen equilibrium was determined. When 0.1% cystine was present, so that the animals were ingesting about 10 mg of cystine per day, they were approximately in nitrogen equilibrium when receiving 0.04%, or 4.2 mg of methionine per day; when no cystine was added, 0.14%, or 14.6 mg methionine per day, was just sufficient to establish equilibrium. Since the growing rat uses both the L- and the D- forms of methionine (Jackson and Block, '33; Rose, '38; Wretlind and Rose, '50), it is assumed that the adult rat possesses the same ability.

The finding that such a large proportion of the methionine-cystine requirement could be furnished as cystine was unexpected, since earlier experiments (Womack and Rose, '41) had indicated that for growth under the conditions of that study

TABLE 2

*DL-methionine requirement for the establishment of nitrogen equilibrium in adult male rats on rations containing 9 essential amino acids with either diammonium citrate (diet 1) or 10 non-essential amino acids (diet 2)*

| RATION | PERIODS TOTAL NO. OF 4-DAY | AVERAGE DAILY INTAKE | | | | AVERAGE WEIGHT CHANGE [1] | AVERAGE NITROGEN BALANCE AND STANDARD ERROR [2] |
|---|---|---|---|---|---|---|---|
| | | Food | Methionine | Cystine | Nitrogen | | |
| | | *gm* | *mg* | *mg* | *mg* | *gm/day* | *mg/day* |
| Diet 1 | 23 | 14.0 | 28.0 | 0 | 64.5 | — 0.3 | + 6.9 ± 1.9 |
| Diet 1A (no methionine) | 24 | 11.9 | 0 | 0 | 50.0 | — 2.6 | — 35.2 ± 1.5 |
| Diet 1A + 0.12% DL-methionine | 29 | 10.4 | 12.5 | 0 | 49.0 | — 0.6 | — 4.8 ± 1.5 |
| Diet 1A + 0.14% DL-methionine | 30 | 10.4 | 14.6 | 0 | 48.7 | — 0.3 | + 0.1 ± 1.3 |
| Diet 1A + 0.28% DL-methionine | 28 | 11.5 | 32.2 | 0 | 52.6 | + 0.3 | + 11.8 ± 1.4 |
| Diet 2 | 24 | 13.3 | 26.6 | 13.3 | 61.1 | — 0.5 | + 6.0 ± 1.8 |
| Diet 2A (no methionine) | 28 | 9.6 | 0 | 9.6 | 42.0 | — 2.0 | — 14.2 ± 1.2 |
| Diet 2A + 0.04% DL-methionine | 29 | 10.6 | 4.2 | 10.6 | 47.6 | — 0.4 | — 2.2 ± 2.0 |
| Diet 2A + 0.05% DL-methionine | 28 | 11.4 | 5.7 | 11.4 | 51.2 | + 0.3 | + 7.1 ± 1.2 |

[1] Average starting weights of the animals on diet 1, 362.8 gm; on diet 2, 361.3 gm.

[2] Standard error of the mean, $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$.

only about one-sixth of the methionine-cystine requirement could be satisfied by cystine. A second study was conducted to investigate the effect of the non-essential amino acids on the amino acid requirement. This time 0.2 instead of 0.1% cystine was included in each ration in which the methionine level was reduced. The results are presented in table 3. When methionine was fed at a level of 0.03%, or 3.2 mg per day, the animals were in nitrogen equilibrium. Since this was true on diet 1 from which the non-essential amino acids other than cystine were absent, as well as on diet 2, the results indicate that cystine is the only non-essential amino acid which influences the methionine requirement. A test in which 50 μg of vitamin $B_{12}$ were added per kilogram of ration showed no effect of the vitamin on methionine requirement as reflected in the nitrogen balances.

In the first experiment, a daily methionine intake of 14.6 mg maintained the animals in equilibrium when no cystine was present; in the second, 3.2 mg were sufficient when more than 20 mg per day of L-cystine were being ingested. From these experiments it appears that only about one-fourth of the methionine requirement for maintenance of nitrogen equilibrium in these animals must be supplied as methionine, and that the rest may be furnished as either cystine or methionine. Higher levels of cystine were not studied, so that this is not evidence as to the maximum amount of methionine that can be replaced by cystine. Certainly the point of diminishing returns was reached when 10.6 mg of cystine reduced the methionine requirement from 14.6 mg to 4.2, and an additional 10.8 mg reduced it only to 3.2 mg.

The results of the experiment to study the effect of the non-essential amino acids on the isoleucine requirement are presented in table 4. When isoleucine was omitted, the weight losses and negative nitrogen balances of the animals on the two diets were similar. The addition of 0.24% DL-isoleucine caused gains in weight and positive nitrogen balances in both groups of animals. When the amount of isoleucine in the ration was decreased to 0.22%, the animals were approximately in nitro-

TABLE 3

*DL-methionine requirement for the establishment of nitrogen equilibrium in adult male rats on rations containing 9 essential amino acids with either diammonium citrate plus 0.2% L-cystine (diet 1B) or 10 non-essential amino acids including 0.2% L-cystine (diet 2B)*

| RATION | TOTAL NO. OF 4-DAY PERIODS | AVERAGE DAILY INTAKE | | | | AVERAGE WEIGHT CHANGE [1] | AVERAGE NITROGEN BALANCE AND STANDARD ERROR |
|---|---|---|---|---|---|---|---|
| | | Food | Methionine | Cystine | Nitrogen | | |
| | | *gm* | *mg* | *mg* | *mg* | *gm/day* | *mg/day* |
| Diet 1 | 24 | 12.9 | 25.7 | 25.7 | 58.2 | — 0.9 | — 6.6 ± 1.5 |
| Diet 1B (no methionine) | 30 | 10.3 | 0 | 20.6 | 49.2 | — 1.3 | — 14.6 ± 0.7 |
| Diet 1B + 0.02% DL-methionine | 30 | 10.7 | 2.2 | 21.4 | 51.4 | — 0.4 | — 5.6 ± 1.1 |
| Diet 1B + 0.03% DL-methionine | 30 | 10.5 | 3.2 | 21.0 | 48.2 | — 0.3 | + 0.2 ± 0.7 |
| Diet 1B + 0.04% DL-methionine | 29 | 11.0 | 4.4 | 22.0 | 52.8 | + 0.3 | + 5.7 ± 0.9 |
| Diet 2 | 24 | 15.1 | 30.3 | 30.3 | 70.1 | — 0.1 | + 3.5 ± 1.4 |
| Diet 2B (no methionine) | 30 | 10.3 | 0 | 20.7 | 46.5 | — 1.6 | — 16.6 ± 0.7 |
| Diet 2B + 0.02% DL-methionine | 30 | 11.1 | 2.2 | 22.2 | 49.9 | — 0.4 | — 8.1 ± 1.1 |
| Diet 2B + 0.03% DL-methionine | 30 | 10.7 | 3.2 | 21.4 | 48.2 | — 0.2 | + 1.2 ± 1.0 |
| Diet 2B + 0.04% DL-methionine | 30 | 11.6 | 4.7 | 23.3 | 52.5 | + 0.4 | + 6.3 ± 1.0 |
| Diet 2B + 0.03% DL-methionine + 50 μg $B_{12}$ per kg of ration | 30 | 10.3 | 3.1 | 20.6 | 47.0 | — 0.3 | + 1.9 ± 1.0 |

[1] Average starting weights of the animals on diet 1, 392.3 gm; on diet 2, 392.8 gm.

gen equilibrium and both groups had an average weight loss of 0.5 gm per day. These findings indicate that after a period of isoleucine deprivation, about 22 mg of DL-isoleucine are sufficient to maintain adult rats in approximate nitrogen equilibrium. The absence of the non-essential amino acids does not appear to increase the amount of isoleucine required for the establishment of nitrogen equilibrium. If it is assumed that adult rats, like young rats (Rose, '38), are unable to utilize D-isoleucine, then the amount of L-isoleucine required to maintain the animals in nitrogen equilibrium on these rations is about 11 mg per day.

Results of the study of the influence of the non-essential amino acids on the threonine requirement of the adult animal are shown in table 5. When threonine is withdrawn from the ration, there are large losses in weight and large losses of nitrogen. With the addition of 0.20% DL-threonine, the nitrogen losses are considerably reduced and when the DL-threonine content of the ration is increased to 0.24%, giving a DL-threonine intake of approximately 20 mg per day, positive nitrogen balance is established. Since there were no statistically significant differences between the nitrogen balances with the two rations, it is apparent that the absence of the non-essential amino acids does not increase the threonine requirement. The rat utilizes only the naturally-occurring form of threonine (West and Carter, '38); therefore under these experimental conditions about 10 mg of L-threonine per day are sufficient to maintain the animals in nitrogen equilibrium.

The animals of all groups were fed ad libitum and the caloric intakes, which were high initially (57 to 70 calories per day), became stabilized at a lower level during the periods of amino acid supplementation which followed deprivation. It will be observed that for each dietary level of amino acid studied, the average food consumption and hence the average nitrogen and caloric intake were not appreciably different on the two rations being compared. Swanson ('51), in her study of the effect of caloric intake on the nitrogen balance of rats fed protein-free rations, found no appreciable effect on nitrogen balance on a

TABLE 4

*DL-isoleucine requirement for the establishment of nitrogen equilibrium in adult male rats on rations containing 9 essential amino acids with either diammonium citrate (diet 1) or 10 non-essential amino acids (diet 2)*

| RATION | TOTAL NO. OF 4-DAY PERIODS | AVERAGE DAILY INTAKE | | | AVERAGE WEIGHT CHANGE [1] | AVERAGE NITROGEN BALANCE AND STANDARD ERROR |
|---|---|---|---|---|---|---|
| | | Food | Isoleucine | Nitrogen | | |
| | | *gm* | *mg* | *mg* | *gm/day* | *mg/day* |
| Diet 1 | 24 | 13.1 | 52.3 | 57.6 | — 0.6 | — 3.3 ± 2.3 |
| Diet 1C (no isoleucine) | 29 | 9.4 | 0 | 40.3 | — 2.6 | — 35.2 ± 1.7 |
| Diet 1C + 0.24% DL-isoleucine | 27 [2] | 13.1 | 31.5 | 59.0 | + 1.0 | + 8.2 ± 1.2 |
| Diet 1C + 0.22% DL-isoleucine | 25 | 9.8 | 21.6 | 43.9 | — 0.5 | — 0.5 ± 0.7 |
| Diet 2 | 24 | 13.7 | 54.8 | 59.0 | — 0.1 | — 2.5 ± 2.2 |
| Diet 2C (no isoleucine) | 30 | 8.6 | 0 | 36.3 | — 2.8 | — 38.6 ± 2.3 |
| Diet 2C + 0.24% DL-isoleucine | 30 | 13.2 | 31.6 | 58.3 | + 1.0 | + 3.7 ± 1.2 |
| Diet 2C + 0.22% DL-isoleucine | 30 | 10.5 | 23.1 | 45.0 | — 0.5 | — 2.0 ± 0.9 |

[1] Average starting weights of the animals on diet 1, 370.7 gm; on diet 2, 370.3 gm.

[2] One rat died after three periods, from a respiratory infection.

TABLE 5

*DL-threonine requirement for the establishment of nitrogen equilibrium in adult male rats on rations containing 9 essential amino acids with either diammonium citrate (diet 1) or 10 non-essential amino acids (diet 2)*

| RATION | TOTAL NO. OF 4-DAY PERIODS | AVERAGE DAILY INTAKE | | | AVERAGE WEIGHT CHANGE [1] | AVERAGE NITROGEN BALANCE AND STANDARD ERROR |
|---|---|---|---|---|---|---|
| | | Food | Threonine | Nitrogen | | |
| | | *gm* | *mg* | *mg* | *gm/day* | *mg/day* |
| Diet 1 | 24 | 13.1 | 52.5 | 60.3 | −0.7 | +2.0 ± 1.8 |
| Diet 1D (no threonine) | 27 | 10.7 | 0 | 43.2 | −2.3 | −36.1 ± 1.7 |
| Diet 1D + 0.20% DL-threonine | 27 | 8.4 | 16.8 | 35.9 | −0.7 | −4.9 ± 1.0 |
| Diet 1D + 0.24% DL-threonine | 25 | 8.5 | 20.5 | 38.5 | −0.4 | +2.2 ± 0.7 |
| Diet 1D + 0.26% DL-threonine | 25 | 8.8 | 22.8 | 40.4 | −0.3 | +4.8 ± 0.8 |
| Diet 2 | 22 | 15.0 | 60.2 | 68.0 | −0.2 | +8.1 ± 2.1 |
| Diet 2D (no threonine) | 24 | 10.5 | 0 | 43.2 | −2.6 | −39.5 ± 2.0 |
| Diet 2D + 0.20% DL-threonine | 28 | 8.0 | 16.0 | 33.4 | −0.5 | −4.0 ± 1.0 |
| Diet 2D + 0.24% DL-threonine | 25 | 8.1 | 19.4 | 34.9 | −0.1 | +1.5 ± 1.3 |
| Diet 2D + 0.26% DL-threonine | 25 | 8.8 | 22.9 | 39.7 | −0.1 | +5.9 ± 1.1 |

[1] Average starting weights of the animals on diet 1, 386.7 gm; on diet 2, 380.7 gm.

high fat diet until the caloric intake had been reduced to 12.2 calories per day, and on a low fat diet to 24.5 calories per day. Even at the lowest level of food intake (in the threonine study), 37 calories per day were ingested by the animals, a level well above that at which Swanson found caloric intake to affect nitrogen balances. Munro ('51) concluded that there is a dynamic relationship between nitrogen balance and energy intake, so that a change in one brings about an alteration in the other. Only in the isoleucine study reported here was there any

TABLE 6

*Daily amino acid requirements for the adult rat*

| AMINO ACID [1] | Mg/100 cm² | | Mg/kg$^{3/4}$ | | Mg/kg rat | |
|---|---|---|---|---|---|---|
| | Benditt et al. | Present authors | Nasset et al. | Present authors | Mitchell | Present authors |
| Isoleucine | 12.9 | 2.7 [2] | | | 64.0 | 34.0 [2] |
| Methionine-cystine | | | | | 58.8 | |
| Methionine (no cystine) | | 4.2 | 40 | 40.5 | | 56.9 |
| Methionine (5.5 mg cystine per day) | 5.3 | 2.6 [2] | | | | |
| Threonine | 5.1 | 2.8 [2] | 20.1 | 28.6 | 37.5 | 39.3 [2] |

[1] Values given in terms of "utilizable" forms.

[2] Average of values obtained on diets 1 and 2.

[3] Calculated value assuming a straight line relationship between methionine requirement and cystine intakes of 0 and 10 mg per day.

appreciable variation in the caloric intake between two levels of amino acid feeding. This does not affect comparison between the same level on the two diets, but the decreases in the nitrogen balances found when the isoleucine level was changed from 0.24% to 0.22% may be the result not only of decreased isoleucine but also of decreased caloric intakes.

Other investigators have studied or calculated the amino acid requirements of the adult rat. Their results have been reported either as requirements for a 325-gm rat (Mitchell, '50), as milligrams per day per kg$^{3/4}$ (Nasset and Anderson, '51; Nasset, Anderson and Siliciano, '51), or as milligrams per 100 cm² per

day (Benditt et al., '50). Table 6 summarizes the results of these investigators in comparison with those of the present study. For this table, Mitchell's estimates have been recalculated as milligrams per kilogram of rat per day. There is remarkably good agreement between the methionine requirement determined in the present investigation and the values of Mitchell and of Nasset and Anderson. The threonine requirement indicated by this study agrees well with the estimate made by Mitchell but is somewhat lower than the value reported by Benditt and co-workers and higher than that found by Nasset, Anderson and Siliciano. There is considerable divergence among the several values for isoleucine.

## SUMMARY

Experiments have been conducted to determine the influence of the non-essential amino acids on the amounts of methionine, isoleucine and threonine required to maintain adult male rats in nitrogen equilibrium. Cystine was the only non-essential amino acid which influenced the amount of methionine required for this purpose. In the absence of cystine, an intake of 14.6 mg DL-methionine was required; when the animals received 10 mg of L-cystine, 4.2 mg DL-methionine were sufficient; and when the cystine ingested was increased to 21 mg per day, 3.2 mg DL-methionine were needed for maintenance of nitrogen equilibrium. The isoleucine and threonine requirements, 11 and 10 mg per day, respectively, were unaffected by the non-essential amino acids at the levels of intake used.

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# SOYIN, A TOXIC PROTEIN FROM THE SOYBEAN

## I. INHIBITION OF RAT GROWTH [1]

IRVIN E. LIENER
*Division of Agricultural Biochemistry, University of Minnesota, St. Paul*

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The unique effect of heat in improving the nutritive value of the soybean protein has been of theoretical and practical interest since it was first demonstrated by Osborne and Mendel ('17). Although the presence of a growth-inhibiting substance in raw soybeans has been shown by several investigators (Ham et al., '45; Klose et al., '46), the exact nature of this substance, if indeed it be a single compound, has remained obscure. The evidence that a trypsin inhibitor which has been isolated from raw soybeans (Kunitz, '46) is wholly responsible for growth inhibition has not been unequivocal (see review by Liener, '50). The demonstration that a concentrate of the soybean trypsin inhibitor caused the death of rats when administered by intraperitoneal injection, while the crystalline material was innocuous under the same conditions (Liener, '51), suggested the presence of a toxic substance in raw soybeans.

A more intensive search into the literature has revealed that the recognition of a toxic substance in raw soybeans is not new. Aqueous extracts of raw soybeans were found to be lethal when injected into rabbits (Falk, '14) and dogs (Carnot and Gerard, '19), but since these crude extracts were high in urease activity, death was attributed to the urease content of such preparations. It has been subsequently shown that crystalline urease is in fact highly toxic when injected

[1] Paper 2889, Scientific Journal Series, Minnesota Agricultural Experiment Station.

into rabbits (Tauber and Kleiner, '31). Harris ('15) observed that an albumin-like fraction derived from raw soybeans was toxic when injected into guinea pigs. This preparation, for which he suggested the name "glicin," likewise possessed urease activity, but in addition displayed marked hemagglutinating properties. Since crystalline urease is lacking in hemagglutinating activity (Sumner and Howell, '35), it is probable that the albumin fraction prepared by Harris was a mixture of at least two different proteins. Sjollema ('25) has reported that a soybean fraction, whose method of preparation was not disclosed, displayed hemagglutinating activity and was lethal when injected into rabbits. Okano ('48) has described the preparation of an albumin fraction from raw soybeans which was toxic to rats when injected and retarded growth when fed orally. It is impossible to judge from Okano's report, however, the degree of purity which had been achieved by his isolation procedure.

The isolation and purification of a toxic protein from defatted soybean flour were described in a previous paper (Liener and Pallansch, '52). It is pertinent to note that in addition to being toxic to rats, this protein, for which the name "soyin" is now proposed,[2] was characterized by marked hemagglutinating action and was devoid of either urease or antitryptic activity. The purpose of the present investigation was to ascertain by actual feeding trials with rats the extent to which "soyin" might be responsible for the poor nutritive value of unheated soybean oil meal.

[2] For the reasons discussed above, the name "glicin" proposed by Harris ('15) was considered inappropriate. The name "soyin" is suggested to conform with the precedent of basing the names of toxic plant proteins on the botanical classification of the plant from which they are derived. Thus, ricin, abrin and crotin are toxins originating from *Ricinus communis*, *Abrus precatorius*, and *Crotin eluteria*, respectively. Although there is no complete unanimity concerning the botanical name of the soybean, it is generally referred to as *Soya* (also spelled *Soja) max* or *Soya hispida* (Williams, '50). In addition to being toxic, these plant toxins share with soyin the property of being able to agglutinate the red blood cells of various species of animals.

## EXPERIMENTAL

### *Preparation of soyin on a large scale*

The requirement of large amounts of soyin for the feeding experiments to be described led to the adoption of the following modifications of the previously published procedure for the preparation of this material (Liener and Pallansch, '52). Fifteen hundred grams of defatted soybean flour [3] were suspended in 18 l of distilled water at room temperature. With constant stirring the pH was adjusted to 6.7 with 5 N NaOH, and the stirring was continued for an additional hour. The suspension was acidified with 6 N HCl to pH 4.6, and the curd was allowed to settle overnight at 4°C. Most of the clear supernatant fluid could be siphoned off, and the remainder of the water extract was removed by filtration on 10-inch Buchner funnels with the aid of suction. To each 10 l of water extract were gradually added, while stirring, 3 kg of $(NH_4)_2SO_4$. The precipitate which formed was removed by filtration and discarded, and 1 kg $(NH_4)_2SO_4$ was added to the clear yellow filtrate. After allowing the precipitate to settle overnight, most of the supernatant fluid was siphoned off, and the precipitate was centrifuged free of the remaining liquor. Sufficient water was added to the precipitate to form a slurry (about 150 ml), which was dialyzed against distilled water at 4°C. for 36 hours. Any insoluble material which precipitated was removed by centrifugation after dialysis. The pH of the solution was adjusted to 4.6 by the dropwise addition of 2 N HCl, and the volume was brought to 200 ml. After the addition of 70 gm $(NH_4)_2SO_4$, the precipitate was centrifuged from the solution and redissolved in 70 ml M/20 phosphate buffer at pH 6.1. The buffered solution was transferred to a mechanical dialyzer [4] and dialyzed against 60% ethanol at

[3] Nutrisoy XXX, Archer-Daniels-Midland Company, Minneapolis, Minnesota. Protein content, 57%.

[4] American Instrument Co., Inc., Silver Spring, Maryland, buffer jar capacity of one liter.

— 5° to — 10°C. for 24 hours. The precipitate formed within the dialysis sack was centrifuged down at — 5°C., dissolved in about 25 ml distilled water, and dialyzed overnight against a large volume of distilled water at 4°C. After centrifuging off any insoluble material that remained, the solution was dried *in vacuo* from the frozen state.

This procedure yielded an average of 2 gm of material per kilogram of soybean flour. Data relating to N content, antitryptic activity, hemagglutinating titer, toxicity (to rats),

TABLE 1

*Properties of the preparations of soyin and soybean trypsin inhibitor (SBTI) used in rat growth studies*

| | SOYIN | SBTI |
|---|---|---|
| Water, % [1] | 12.0 | 4.7 |
| N, % [2] | 16.6 | 15.5 |
| Antitryptic units/mg N × $10^{-3}$ | 0.8 | 22.0 |
| Hemagglutinating titer, μg N [3] | 0.9 | 1.7 |
| Toxic units/mg N × $10^{-3}$ [4] | 123 | 33 |
| Concentration of toxic component, % [5] | 78 | 24 |

[1] Loss of weight at 105° for 6 hours.

[2] Calculated on basis of dry weight.

[3] Highest dilution giving definite agglutination of rabbit erythrocytes.

[4] Reciprocal of the $LD^{50}$ (mg N/kg body wt.) determined with young weanling rats.

[5] Based on electrophoretic measurements: ratio of area of toxic component to total area × 100.

and electrophoretic homogeneity of this preparation of soyin are presented in table 1. The methods employed for these analyses have already been reported (Liener and Pallansch, '52). On the basis of electrophoretic measurements, the toxic component comprises about 78% of the total protein content of the preparation.

### *Animals and rations*

The rats used in this study were of a black strain maintained in this laboratory for many years. In any one experimental series, weanling litter mates weighing 35 to 45 gm

were divided equally between two or three groups, composed of two males and two females in each group. Each animal was housed in an individual screen-bottom cage with access to a ration each kilogram of which contained defatted soybean flour, unheated or autoclaved (15 lb. pressure for 30 minutes), or casein [5] 250 gm, salts (Schultze, '50) 40 gm, corn oil 60 gm, sucrose 650 gm, and vitamin supplements (Schultze, '50) modified to include 40 μg vitamin $B_{12}$. When soybean flour was used as the protein source, each kilogram of the ration was supplemented with 6 gm DL-methionine added at the expense of sucrose. Soyin and crude trypsin, when employed as supplements, were likewise added with an equivalent deduction in sucrose. Food was furnished ad libitum, with the exception of the experiments involving paired feeding, for two weeks during which time daily records were kept of individual food consumption and weight.

## RESULTS

It was considered most desirable to incorporate soyin into a basal ration at a level which approximated the soyin content of the unheated soybean flour. Toxicity studies on a crude water extract showed that the unheated flour contained at least 0.65 toxic units per gram (Liener, '52). Since the soyin preparation used in this study contains approximately 18.0 toxic units per gram (calculated from the value shown in table 1 on the basis of N and water content), it can be readily computed that a ration must be supplemented with at least 0.9% of this preparation in order to make it approximately equivalent in toxic units to one containing 25% unheated soybean flour. Using the growth supported by autoclaved soybean flour as a reference, unheated soybean flour produced a growth inhibition of about 50% (series 1, table 2). When soyin was added to a ration containing autoclaved soybean protein at levels of 0.5% and 1.0%, significant growth inhibition was obtained only at the higher level (series 2 and

[5] Labco.

3, table 2), to the extent of about 26%. If the level of soyin which produced growth inhibition in these experiments, namely 1.0%, can be accepted as a reasonable approximation of the soyin content of a ration containing 25% unheated soybean flour, it follows that soyin per se accounts only in part for the poor growth which is obtained with the unheated flour.

TABLE 2

*Rat growth experiments with soyin and raw or autoclaved soybean flour in the presence or absence of crude trypsin* [1]

| SERIES [2] | PROTEIN SOURCE [2] | SUPPLEMENT | WEIGHT GAIN | | FOOD INTAKE | | GROWTH INHIBITION [6] |
|---|---|---|---|---|---|---|---|
| | | | Mean [4] | "t" value [5] | Mean [4] | "t" value [5] | |
| | | | *gm* | | *gm/day* | | % |
| 1 | Autoclaved soy | None | 43.8 ± 4.2 | | 8.0 ± 0.4 | | |
| | Raw soy | None | 20.5 ± 3.7 | 4.23 | 6.4 ± 0.4 | 3.35 | 53.2 |
| 2 | Autoclaved soy | None | 64.3 ± 5.0 | | 7.7 ± 0.9 | | |
| | Autoclaved soy | 0.5% soyin | 60.3 ± 7.1 | 1.23 | 7.6 ± 0.9 | 0.30 | 6.2 |
| 3 | Autoclaved soy | None | 60.5 ± 5.1 | | 10.4 ± 0.4 | | |
| | Autoclaved soy | 1.0% soyin | 45.0 ± 1.1 | 2.72 | 8.2 ± 0.4 | 3.16 | 25.6 |
| 4 | Casein | None | 68.8 ± 3.6 | | 9.4 ± 0.2 | | |
| | Casein | 1.0% soyin | 56.3 ± 1.1 | 4.40 | 8.1 ± 0.3 | 6.56 | 18.2 |
| 5 | Autoclaved soy | 5% trypsin | 66.8 ± 5.1 | | 9.6 ± 0.7 | | |
| | Raw soy | None | 24.5 ± 8.5 | 11.42 | 6.5 ± 1.3 | 5.31 | 63.3 |
| | Raw soy | 5% trypsin | 48.8 ± 11.1 | 2.46 | 8.6 ± 1.3 | 1.62 | 26.9 |
| 6 | Autoclaved soy | 5% trypsin | 66.0 ± 2.7 | | 9.1 ± 0.2 | | |
| | Autoclaved soy | 5% trypsin + 1.0% soyin | 50.3 ± 3.3 | 3.58 | 7.2 ± 0.5 | 3.98 | 23.8 |
| 7 | Autoclaved soy | None | 43.0 ± 5.6 | | 8.0 ± 1.0 | | |
| | Raw soy | None | 33.3 ± 5.1 | 9.32 | 8.0 ± 1.0 | | 22.6 |
| 8 | Autoclaved soy | 5% trypsin | 44.3 ± 5.9 | | 7.0 ± 0.6 | | |
| | Raw soy | 5% trypsin | 42.3 ± 4.8 | 1.79 | 7.0 ± 0.6 | | 4.5 |
| 9 | Autoclaved soy | None | 50.3 ± 1.7 | | 8.5 ± 0.4 | | |
| | Autoclaved soy | 1.0% soyin | 48.0 ± 1.9 | 1.50 | 8.5 ± 0.4 | | 4.6 |

[1] Trypsin powder (1–110), Pfanstiehl Chemical Co., Waukegan, Ill.

[2] In series 1–6, animals were fed ad libitum. In series 7–9, the food intake of the control group was restricted to that of the experimental group.

[3] The group listed first within each series constitutes the control.

[4] The mean of each group (4 animals) ± the standard error of the mean over a period of two weeks.

[5] Calculated according to Snedecor ('46) on the basis of paired comparisons between litter mates of the same initial weight (± 1 gm) and sex distributed in the control and experimental groups: "t" values $> 2.45$ indicate a difference from the control significant at a level of $< 0.05$, and "t" values $> 3.71$ indicate a difference significant at a level $< 0.01$.

[6] Per cent difference in gain in weight between control and experimental groups.

Growth inhibition was also evident to a somewhat lesser degree when 1.0% soyin was added to a diet containing 25% casein (series 4, table 2). The higher protein content of the casein ration may account for the less effective growth inhibition produced by soyin in this instance. A sufficient quantity of soyin was not available to permit a comparison of its effect on growth in a ration containing casein at a protein level equivalent to that of a ration containing 25% soybean flour.

Borchers and Ackerson ('51) have reported that crude trypsin powder at a level of 5% completely counteracts the growth inhibition obtained with raw soybean oil meal. The results of series 5, table 2, show that although crude trypsin does improve the nutritive value of the unheated soybean flour, the restoration of growth was not complete. In fact, this difference in growth response, which persists in the presence of crude trypsin (26.9%), was strikingly similar to that obtained when 1.0% soyin was added to the autoclaved protein (25.6%). It will also be noted that crude trypsin did not appreciably alter the growth inhibition induced by 1.0% soyin (compare series 3 and 6). This is a further indication that the growth inhibitory effect of raw soybean flour which remained after supplementation with trypsin could have been due to the growth-retarding effect evoked by soyin.

The food consumption data summarized in table 2 show that growth inhibition was generally accompanied by a significant decrease in the level of food intake. To determine the extent to which these differences in food intake might be responsible for differences in growth response, the paired feeding studies shown in series 7 to 9, table 2, were conducted. The daily food consumption of each of the rats in the control group was restricted to that of its litter mate in the experimental group. When the daily food intake of rats receiving the autoclaved soybean flour was restricted to that of animals receiving the unheated flour, growth inhibition was reduced to about one-half that previously observed with ad libitum feeding. In the case of rats receiving 1.0% soyin or raw soybean flour plus trypsin, equalization of the food

intake failed to reveal any significant differences in growth such as had been noted with unrestricted feeding. It would appear, therefore, that the growth-limiting effect of soyin is simply a manifestation of a lowered intake of food, an explanation which may also account for a substantial portion of the growth inhibition obtained with raw soybean flour. The growth inhibition with raw soybean flour which remained unaffected by trypsin would also seem to be the result of decreased food consumption, an effect which is presumably due to soyin.

Since the preparation of soyin used in this study retained traces of antitryptic activity (see table 1), it became necessary to rule out the latter as a cause of the growth inhibition observed here. A crude concentrate of the soybean trypsin inhibitor (SBTI), prepared according to the method of Klose et al. ('46), was incorporated into a ration containing autoclaved soybean flour or casein at levels of 1% and 2% (with an equivalent deduction in sucrose), so as to impart to the ration a degree of antitryptic activity far in excess of that provided by the soyin preparation. The properties of SBTI and its effect on rat growth are presented in tables 1 and 3, respectively. Also included in table 3 for comparative purposes are the results obtained with soyin. SBTI at a level of 1%, which provided 27 times the antitryptic activity of a ration containing 1.0% soyin, was relatively ineffective as a growth inhibitor. When the level of SBTI was increased to 2%, significant growth inhibition was obtained in spite of the fact that the number of toxic units contained in this ration was only slightly in excess of the toxic units provided by 0.5% soyin, which is non-inhibitory to growth. This would indicate that some other growth-inhibiting factor (antitrypsin?) in addition to soyin is present in SBTI.

## DISCUSSION

All the data reported here are consistent with the view that the poor growth obtained with raw soybean flour is a

TABLE 3

*Comparison of toxicity, antitryptic activity, and growth inhibitory effect of soyin and a crude trypsin inhibitor (SBTI) preparation*

| SERIES [1] | PROTEIN SOURCE [2] | SUPPLEMENT | TOXICITY [3] | ANTITRYPTIC ACTIVITY [3] | WEIGHT GAIN | | GROWTH INHIBITION [6] |
|---|---|---|---|---|---|---|---|
| | | | | | Mean [4] | "t" value [5] | |
| | | | *Units/100 gm ration* | *Units/100 gm ration* | *gm* | | % |
| 2 | Autoclaved soy | None | 0 | 0 | 64.3 ± 5.0 | | |
| | Autoclaved soy | 0.5% soyin | 9.0 | 0.06 | 60.3 ± 7.1 | 1.23 | 6.2 |
| 3 | Autoclaved soy | None | 0 | 0 | 60.5 ± 2.7 | | |
| | Autoclaved soy | 1.0% soyin | 18.0 | 0.12 | 45.0 ± 1.1 | 2.72 | 25.6 |
| 4 | Casein | None | 0 | 0 | 68.8 ± 3.6 | | |
| | Casein | 1.0% soyin | 18.0 | 0.12 | 56.3 ± 1.1 | 4.40 | 18.2 |
| 10 | Autoclaved | None | 0 | 0 | 55.0 ± 4.2 | | |
| | Autoclaved | 1% SBTI | 4.9 | 3.24 | 54.5 ± 2.0 | 0.50 | 0.9 |
| | Autoclaved | 2% SBTI | 9.8 | 6.48 | 40.0 ± 5.1 | 3.29 | 23.8 |
| 11 | Casein | None | 0 | 0 | 69.8 ± 2.3 | | |
| | Casein | 1% SBTI | 4.9 | 3.24 | 68.0 ± 2.5 | 1.60 | 2.5 |
| | Casein | 2% SBTI | 9.8 | 6.48 | 59.0 ± 2.7 | 2.56 | 15.4 |

[1] All animals in these experiments were fed ad libitum.

[2] The group listed first within each series constitutes the control.

[3] Calculated from values taken from table 1 based on N and water content.

[4] The mean of each group (4 animals) ± the standard error of the mean over a period of two weeks.

[5] Calculated according to Snedecor ('46) on the basis of paired comparisons between litter mates of the same initial weight (± 1 gm) and sex distributed in the control and experimental groups: "t" values $> 2.45$ indicate a difference from the control significant at a level of $< 0.05$, and "t" values $> 3.71$ indicate a difference significant at a level $< 0.01$.

[6] Per cent difference in gain in weight between control and experimental groups.

function of two effects which can be differentiated on the basis of the response to crude trypsin. Although the growth-retarding effect which is counteracted by crude trypsin would logically appear to be due to antitrypsin, it has been reported by Borchers and Ackerson ('51) that the tryptic activity of crude trypsin is not responsible for this reversal of growth inhibition. Hence, the mechanism whereby crude trypsin exerts its beneficial effect on raw soybeans is still obscure.

The present evidence strongly suggests that the growth-inhibiting effect which is not counteracted by trypsin is due to the soyin content of raw soybeans. The effect of soyin ingestion appears to be an enforced limitation in food intake with a consequent impairment of growth. Whether soyin simply affects the palatability of the ration or creates some metabolic disturbance which leads to diminished appetite cannot be answered at the present time. It is interesting to note that Osborne and Mendel ('17) attributed the poor growth of rats on raw soybean meal to their failure to consume this meal as readily as the heated meal.

In experiments with young chicks fed varying proportions of raw to cooked soybean meals, Almquist and Merritt ('52) observed that the growth inhibition associated with raw soybean meal was almost fully developed when one-fourth of the protein was supplied in the raw form. This effect they explained on the basis of the soybean antitrypsin, which inactivates trypsin but permits the continued functioning of the remaining proteolytic system. They attributed, however, a further slight inhibition of growth with increased proportions of raw soybean meal to a second effect, which they believed to be due to a decreased availability of other nutrients. It is not unlikely that this second effect may be the result of the soyin content of the raw meal as the proportion of the latter is increased in the ration.

In the present study with rats, about half the growth impairment obtained with raw soybean flour may be attributed to its soyin content, and the remainder to an effect which is counteracted by crude trypsin. Thus, it will be recalled from

the data of table 2 that the growth inhibition obtained with the raw soybean flour alone was 53% (series 1), of which 24 to 26% was due to soyin (series 3 and 6). Furthermore, in the presence of crude trypsin, growth inhibition on raw soybean flour was reduced to approximately one-half the value obtained in the absence of trypsin.

These discrepancies in the relative magnitude of the two effects reported by Almquist and Merritt ('52) for chicks and by the present author for rats may be the result of a species difference. It is known, for instance, that with chicks marked differences in the digestibility of the proteins of raw and autoclaved soybean oil meal are obtained (Evans et al., '47), whereas in rats the differences in digestibility are slight (Melnick et al., '46; Desikachar and De, '50). This would indicate that an inhibition of intestinal proteolysis by soybean antitrypsin may not play as important a role in the utilization of soybean protein by the rat as it does in the case of the chick.

## SUMMARY

The procedure for preparing purified soyin on a large scale from defatted soybean flour is described in detail. When soyin was fed to young rats at a level of 1% in a diet containing autoclaved soybean protein or casein, growth was inhibited 26% and 18%, respectively. Crude trypsin was not effective in counteracting this inhibition of growth. Equalization of food intake failed to reveal any significant differences in the growth of rats receiving diets with or without soyin. On the basis of comparable experiments with raw soybean meal in the presence and absence of crude trypsin, it is estimated that about half of the growth-inhibiting effect of raw soybean meal is due to its soyin content and the remainder to an effect which can be counteracted by crude trypsin. Growth impairment by soyin was attributed to a decrease in the quantity of food consumed.

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# THE VITAMIN $B_{12}$ REQUIREMENT OF MINK[1]

W. L. LEOSCHKE, R. J. LALOR[2] AND C. A. ELVEHJEM
*Department of Biochemistry, University of Wisconsin, Madison*

TWO FIGURES

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Studies on the nutritional requirements of mink have shown that on a purified diet these animals require three unidentified factors present in liver and one unidentified factor present in desiccated hog intestinal mucosa (Schaefer, Tove, Whitehair and Elvehjem, '48; Tove, Lalor and Elvehjem, '50a). These 4 factors may be listed as follows: (1) methanol-soluble (extract) factor, (2) methanol-insoluble (residue) factor, (3) hog mucosa factor and (4) a spleen or liver factor not present in the extract and residue fractions of liver.

Recent studies by Tove, Lalor and Elvehjem ('50b) have indicated a relationship between the extract factor required by the mink and vitamin $B_{12}$. The extract factor is found in liver, whole milk powder and fish solubles, which are excellent sources of vitamin $B_{12}$. The extract factor is soluble in water, phenol and 60% methanol and insoluble in acetone, *n*-butanol and three-fourths saturated ammonium sulfate.

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Part of this work was presented before the Division of Agricultural and Food Chemistry, 121st meeting of the American Chemical Society, March 30–April 3, 1952, at Milwaukee, Wisconsin.

We wish to acknowledge our indebtedness to Merck and Company, Inc., Rahway, New Jersey, for the crystalline vitamins.

[2] Present address: Nutritional Research Department, Archer-Daniels-Midland Company, Minneapolis, Minnesota.

Vitamin $B_{12}$ has similar properties. The methanol extract, which is active at a level of less than 1 ml per day, contains 2 μg of vitamin $B_{12}$ per milliliter. Although early studies with low levels of vitamin $B_{12}$ (daily intramuscular injection of 1 μg) had shown that vitamin $B_{12}$ would not replace the extract factor, later work has shown that high levels of vitamin $B_{12}$ (10 and 20 μg per day) are effective in curing the extract deficiency. Considering the striking correlation between the sources and properties of the extract factor and vitamin $B_{12}$ and the results of vitamin $B_{12}$ supplementation, Tove and co-workers postulated that the extract factor required by the mink might be another form of vitamin $B_{12}$, as yet not isolated.

Since the responses of mink to different supplements are dependent upon the relative state of deficiency of the animal, it was thought advisable to study more thoroughly the relationship of the extract factor to vitamin $B_{12}$. The data presented in this report demonstrate that the extract factor required by the mink is vitamin $B_{12}$. Data on the influence of the type of carbohydrate in the diet on the vitamin $B_{12}$ requirement of the mink are also given.

## EXPERIMENTAL AND RESULTS

Adult male mink were used in these studies and were given the same basal diet as was employed in the earlier studies. This basal diet consisted of sucrose 66, casein [3] 19, cottonseed oil 11, and salts IV (Phillips and Hart, '35) 4%. Each 100 gm of ration were supplemented with 0.2 mg thiamine HCl, 0.2 mg pyridoxine HCl, 0.4 mg riboflavin, 1.5 mg Ca pantothenate, 4.0 mg nicotinic acid, 100 mg choline, 25 mg *i*-inositol, 50 mg *p*-aminobenzoic acid, 0.5 mg 2 methyl 1-4 naphthoquinone, 0.1 mg pteroylglutamic acid and 0.025 mg biotin. Haliver oil fortified with α-tocopherol acetate and vitamin $D_3$ was added so that 100 gm of ration contained 1,200 I.U. vitamin A, 120 I.U. vitamin D and 4 mg α-tocopherol acetate.

[3] General Biochemicals, Inc.

Within 8 to 10 months after being placed on the experimental diet, the mink developed the extract deficiency, which is characterized by anorexia, loss of body weight and severe fatty degeneration of the liver. With the onset of this extract deficiency the mink were given the desired supplements orally until they started to eat again. The supplements were then mixed daily with the purified diet. Anorexia has been one

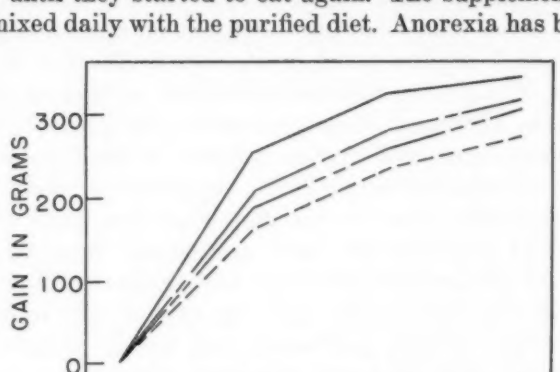


Fig. 1 Responses of mink with the extract deficiency to methanol extract and vitamin $B_{12}$ supplements.

——————— liver extract (9)
——— - - ——— 10 and 20 $\mu$g vitamin $B_{12}$ daily (10)
——— - ——— 3 and 5 $\mu$g vitamin $B_{12}$ daily plus methionine and cystine (6)
- - - - - - - - - 2 and 3 $\mu$g vitamin $B_{12}$ daily (3)

(The number of animals used in each group is indicated by the number included in parentheses after each group).

of the greatest difficulties encountered in obtaining responses with vitamin $B_{12}$ supplementation. The responses to vitamin $B_{12}$ were obtained only after three to 11 days of repeated force-feeding of the deficient mink. The mink were force-fed by means of a slurry of the ration delivered from a Mohr pipette.

The weight responses of deficient mink to different levels of vitamin $B_{12}$ are presented in figure 1. The average re-

sponse of mink to the methanol extract of liver obtained by Tove, Lalor and Elvehjem ('50b) is also given for comparison. Although the weight responses of mink to supplements of vitamin $B_{12}$ are not as rapid as the typical responses to methanol extract of liver, the average gains after 12 weeks' supplementation are approximately equal. In the case of three mink showing suboptimum weight responses (120 gm in 7 weeks) to 10 μg of vitamin $B_{12}$ per day, the addition of 0.12% methionine and 0.06% cystine to the diet resulted in additional weight increases (200 gm in 7 weeks). Since methionine and cystine seemed to facilitate the responses of mink to vitamin $B_{12}$ supplements, these amino acids were also added to the diet when low levels of vitamin $B_{12}$ (3 and 5 μg per day) were used. Recently, three mink with the extract deficiency have responded to supplements of 2 μg (one mink) and 3 μg (two mink) of vitamin $B_{12}$ per day without methionine and cystine supplementation. Thus, although methionine and cystine may facilitate additional weight gains by mink responding suboptimally to vitamin $B_{12}$ supplements, some mink will give optimum responses (weight gains equal to those achieved with methanol extract of liver) without methionine and cystine supplementation.

The vitamin $B_{12}$ requirement of mink is apparently related to the type of carbohydrate in the diet. Mink placed on a purified basal diet in which dextrin is the source of carbohydrate develop a vitamin $B_{12}$ deficiency sooner than mink placed on a similar purified basal diet in which sucrose is the carbohydrate source. If, with the onset of this deficiency, the dextrin basal diet is replaced by the sucrose basal diet, there is an immediate increase in weight and apparent recovery from the vitamin $B_{12}$ deficiency. However, the response to the change in diet is only temporary and within three to 7 months the mink again develop a vitamin $B_{12}$ deficiency. Weight responses of vitamin $B_{12}$-deficient mink on the dextrin basal diet to supplements of vitamin $B_{12}$ (4

mink) or to a change in the diet from the dextrin basal to the sucrose basal diet (6 mink) are shown in figure 2.

Since a change in the type of carbohydrate in the diet from dextrin to sucrose seemed to alleviate the vitamin $B_{12}$ deficiency temporarily, we decided to study possible differences in vitamin $B_{12}$ synthesis by the intestinal flora of mink

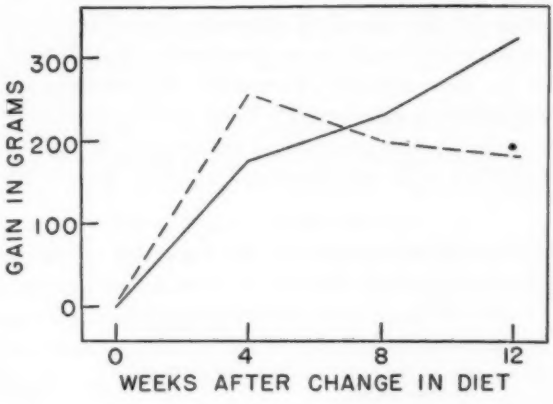


Fig. 2 Responses of mink on the dextrin basal diet to (a) vitamin $B_{12}$ supplementation or (b) to a change from the dextrin basal to the sucrose basal diet.

———— 5 μg vitamin $B_{12}$ daily (4).

– – – – – – – – change from the dextrin basal to the sucrose basal diet.

* after this point the animals continue to lose weight unless given vitamin $B_{12}$ therapy.

(The number of animals used in each group is indicated by the number included in parentheses after each group.)

on dextrin and sucrose basal diets. Vitamin $B_{12}$ analyses were made on 24-hour fecal samples from mink that had been on the dextrin and sucrose basal diets for three months. The samples were collected at 30-minute intervals and immediately placed in 50% ethyl alcohol to prevent further synthesis of vitamin $B_{12}$ after defecation. The samples were then analyzed for vitamin $B_{12}$ by *Lactobacillus leichmannii* assay [4]

[4] We are indebted to U. J. Lewis for the vitamin $B_{12}$ analyses of prepared mink fecal samples.

(Thompson, Dietrich and Elvehjem, '50). The results of these analyses are shown in table 1. It may be seen that the average vitamin $B_{12}$ fecal excretion of mink on the sucrose basal diet was over 6 times that of mink on the dextrin basal diet.

## DISCUSSION

From the data presented it is evident that the extract factor required by the mink is vitamin $B_{12}$. The weight responses of deficient mink to supplements of different levels of vitamin $B_{12}$ were equal to the weight responses to methanol extract supplements obtained by Tove and co-workers ('50b). Although earlier workers had not succeeded in replacing the methanol extract with low levels of vitamin $B_{12}$ (daily intramuscular injection of 1 μg), responses of deficient mink to oral supplements of 2 and 3 μg of vitamin $B_{12}$ per day have now been obtained. The more satisfactory results obtained now may be due to improved techniques of force-feeding the deficient mink.

TABLE 1

*Fecal vitamin $B_{12}$ excretion of mink on the dextrin and sucrose basal diets*

| NUMBER OF MINK | DIET | FECAL VITAMIN $B_{12}$ EXCRETION [1] | RANGE |
|---|---|---|---|
| | | *μg per 24 hr.* | *μg* |
| 5 | Sucrose basal | 0.98 | 0.38–1.60 |
| 5 | Dextrin basal | 0.16 | 0.07–0.40 |

[1] Average of two different 24-hour collections.

Fatty degeneration of the liver, one of the characteristic symptoms of the extract deficiency in mink, has not been observed in autopsies of mink which have responded to vitamin $B_{12}$ therapy. Burns and McKibbin ('51) found vitamin $B_{12}$ to be effective in curing fatty degeneration of the liver in the dog. Fatty livers have been seen in mink maintained for prolonged periods on purified diets containing vitamin $B_{12}$. Thus, the fatty liver condition may also be associated with a deficiency of other factors.

As the work with methionine and cystine supplementation is only preliminary, it is difficult to state the exact role of these amino acids in the responses of mink to vitamin $B_{12}$ supplements. Methionine and cystine may act by sparing vitamin $B_{12}$, or their action may be related to the residue factor required by the mink. It should be pointed out that vitamin $B_{12}$ only replaces the extract factor required by the mink. Mink placed on purified diets with adequate supplements of vitamin $B_{12}$ still develop deficiencies of the other unidentified factors.

Apparently mink have a high requirement for vitamin $B_{12}$. Since practical mink diets contain considerable quantities of horsemeat, liver and fish by-products, ranch mink probably receive adequate amounts of vitamin $B_{12}$.

Two types of evidence indicate a greater intestinal synthesis of vitamin $B_{12}$ in the mink when sucrose is the source of carbohydrate in the diet than when dextrin is the dietary carbohydrate source. These are: (1) the responses of vitamin $B_{12}$-deficient mink to a change in the diet from the dextrin basal diet to the sucrose basal diet (fig. 2); and (2) the greater vitamin $B_{12}$ fecal excretion of mink on the sucrose basal diet relative to that of mink on the dextrin basal diet (table 1).

The increased vitamin $B_{12}$ synthesis by the intestinal flora of mink on the sucrose basal diet over that by the intestinal flora of mink on the dextrin basal diet is in contrast to studies with the rat with respect to vitamin $B_{12}$ and other B vitamins. Lewis ('52) has shown that the intestinal synthesis of vitamin $B_{12}$ in the rat is greater when dextrin is the source of carbohydrate than when sucrose is the carbohydrate source. Mannering, Orsini and Elvehjem ('44) have reported a higher fecal excretion of riboflavin by rats on diets in which dextrin is the carbohydrate source than by rats on diets in which the source of carbohydrate is sucrose. Sarma, Snell and Elvehjem ('46) have presented definite evidence of an increased intestinal synthesis of vitamin $B_6$ in

the rat when dextrin is used in place of sucrose in purified diets.

Although dextrin has been shown to favor the intestinal synthesis of B vitamins (including vitamin $B_{12}$) in the rat, our studies show that sucrose and not dextrin favors the intestinal synthesis of vitamin $B_{12}$ in the mink.

## SUMMARY

1. The methanol extract factor (obtained from liver) required by the mink has been shown to be vitamin $B_{12}$.

2. The intestinal synthesis of vitamin $B_{12}$ in the mink is greater on a diet in which sucrose is the carbohydrate source than on a diet in which dextrin is the source of carbohydrate.

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